Project title:	Precolonisation of strawberry runners and tray plant with arbuscular mycorrhizal fungi to manage Verticillium wilt (AHDB Horticulture Studentship)
Project number:	CP 106
Project leader:	Prof Xiangming Xu, NIAB EMR
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Key staff:	PhD student: Benjamin Langendorf
Location of project:	East Malling Research
Industry Representative:	Marion Regan, Hugh Lowe Farms Ltd
Date project commenced:	Oct 2013
Date project completed (or expected completion date):	Oct 2016

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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CONTENTS

GROWER SUMMARY

Headline

1. Arbuscular mycorrhizal fungi (AMF) propagules can colonise strawberry roots when incorporated as a powder layer during the tipping or weaning process under misting conditions, irrespective of specific AMF strains or strawberry cultivars tested. AMF in colonised roots of tray plants can survive several months of cold storage at -2 °C.
2. However, a random mix of AMF inocula with compost in the commercial production of tipping plants resulted in a very low level of AMF colonisation. Thus, to incorporate AMF at this stage, we need to modify the commercial tipping process to ensure that AMF is present as a thin layer just below the surface of compost in each tray cell, increasing the likelihood of AMF colonisation of new roots during misting/weaning.
3. Pre-inoculation of strawberry tipping plants at the weaning stage with AMF does not necessarily improve plant growth during weaning and subsequent early growth before cold storage. Preconisation of plants with AMF did not reduce the wilt development in field conditions. AMF and PGPR (plant growth promoting rhizobacteria) could reduce development of red core (but not crown rot) but this effect is not consistent. We may conclude that using AMF and PGPR alone will not be able to increase the health of tray plants to reduce disease development significantly, especially when plants were either already infected or inoculated with a high dose of pathogen inoculum.
4. Combined with other on-going research, we conclude that using AMF in coir is likely to increase strawberry yield by 5%, particularly under reduced water or nutrient input.

Background

Verticillium wilt of strawberry caused by *Verticillium dahliae* was traditionally controlled in field soils through the use of soil fumigants to reduce quantities of the pathogen to levels which are not economically damaging to subsequent crops. With the gradual reduction in available soil fumigants over recent years, extensive effort has gone into finding alternative control methods including the incorporation of green manures that release volatile fungitoxic compounds (so-called biofumigation), which has shown promise as a component of a disease management strategy.

In a recent Defra Horticulture LINK project (HL0177, SF 77), the incorporation of lavender waste in soils prior to planting effectively reduced Verticillium wilt severity on strawberry. Three key terpenoids were identified as being responsible for the observed suppressive effect. In a subsequent TSB funded project led by East Malling Research (EMR), palletised lavender

waste and microencapsulated terpenoids are being assessed for their efficacy in controlling *V. dahliae.* However, it is almost certain that additional control measures will still be needed. Soil inhabiting arbuscular mycorrhizal fungi (AMF) have been shown to confer a number of benefits to their host plant including enhanced pathogen resistance, so there is merit in investigating this further to ascertain if it offers potential benefits to soil grown strawberry production. In a recently completed project (funded by Interreg), NIAB EMR researchers showed that AMF can increase strawberry yield by up to 10-15% in coir, particularly when plants were subjected either to reduced nutrients or reduced water input (Robinson Boyer et al. 2016).

This project, funded through the AHDB Horticulture studentship scheme, is investigating if pre-colonising strawberry plants with AMF leads to reduced incidence or severity of Verticillium wilt. During the project period, it has been decided, in consultation of the industry, that disease tolerance work should focus on *Phytophthora* instead of wilt because most strawberry production is now in substrate instead of soil.

We have carried out the following research work to investigate: (1) whether AMF could colonise tipping plants under weaning (humid misting) conditions, (2) whether there are interaction between AMF strains and strawberry cultivars in terms of AMF colonisation, (3) whether AMF in colonised roots of tray plants could survive a prolonged period of cold storage, (4) whether AMF colonisation could increase plant tolerance to wilt, (5) whether AMF colonisation could increase plant tolerance to *Phytophthora* diseases, and (6) whether we could establish an *in vitro* system to study the strawberry-AMF-pathogen interaction.

Summary

The work has demonstrated that arbuscular mycorrhizal fungi (AMF) can colonise *in-vitro* produced plantlets in vermiculite and runner-tip produced plants in a peat/perlite based substrate irrespective of AMF strains/species and strawberry cultivars tested. The high moisture conditions during weaning/tipping did not prevent AMF from colonising roots. AMF can survive inside colonised strawberry roots during cold storage at -2°C for several months. Therefore, AMF inoculated during the weaning stage of micro-propagated plants or runner tips can result in a high success rate of mycorrhizal colonisation, independent of plant material size and/or the strawberry cultivar. Commercial AMF inocula may be applied during strawberry tipping without reducing the mycorrhiza viability during subsequent cold storage of pre-colonised plants.

However, pre-inoculation of strawberry transplants with AMF does not necessarily translate to improved plant growth during weaning and subsequent growth prior to cold storage. All AMF species tested on the tissue culture produced plants of the genotype EM1996 increased the

crown diameter of the plantlets, but this increase was only significant with the AMF *Rhizophagus irregularis*. For the runner tip produced plants, the effects of AMF inoculation on crown diameter varied greatly with specific combinations of AMF and cultivars. However, the differences between different AMF treatments were very small and hence are not likely to have any commercial consequences in terms of the yield potential.

The incidence of wilt in potted experiments was very low, despite the fact we used field soil with a moderate level of wilt inoculum. A large field trial showed that AMF pre-inoculation did not improve plant growth after transplantation in soils and did not reduce the incidence of wilt. This result indicates that pre-colonisation of plants with AMF did not offer sufficient advantage over colonisation of plants by resident AMF inoculum in the soils.

Joint inoculation of plants with AMF and PGPR appeared to slow down development of red core rot symptoms. However, these treatments did not affect development of crown rot. This is probably not surprising given the fact we wounded plants before inoculation of a large dose of pathogen inoculum. Thus, improved tolerance offered by AMF and PGPR is not likely to be strong enough to cope with such a high infection pressure. Indeed this finding agrees with the current consensus that AMF/PGPR will only be able to improve plans tolerance against a low to moderate level of disease pressure. Thus, we conclude that use of AMF and PGPR alone will not be able to reduce disease sufficiently when disease pressure exceeds low level.

Use of AMF and PGPR led to increased yield of ca. 5-10% in coir, agreeing with recent studies at NIAB EMR. This is especially the case when strawberry plants were subjected to reduced fertigation regime. Thus combining results from several studies at NIAB EMR, we conclude that use of AMF will on balance lead to increased fruit yield in the range of 5-10% when plants are under reduced fertigation regimes in coir. This increased yield potential may be the main reason for growers to consider using AMF in practice.

In a commercial strawberry nursery, random mix of AMF inocula with compost in the commercial production of tipping plants resulted in a very low level of AMF colonisation (3%), compared to > 60% when a thin layer of AMF was placed just below the surface of compost. Thus, to incorporate AMF at the tipping/weaning stage, we need to modify the commercial production procedure such that a thin layer of AMF is placed below the surface of compost in each cell. It will greatly increase the likelihood of AMF colonisation of new roots during misting/weaning.

Financial Benefits

This is a PhD research project, focusing more on the research aspect. Thus, it does not directly impact on growers' practice. However, this research does suggest that arbuscular mycorrhizal fungi (AMF) can colonise plants during the tipping/weaning process, and can increase

strawberry yield in coir, especially when growers adopt regulated fertigation scheme to save water and nutrient.

- The best estimate of yield increase due to AMF is ca. 5%.

However, the precise financial benefit will depend on the cost of AMF, how plants are to be pre-colonised with AMF and the extent of reduction in fertigation.

However, it should be stressed that pre-colonisation of plants with AMF will not likely result in any noticeable reduction in the level of diseases.

Action Points

- Growers should consider using arbuscular mycorrhizal fungi (AMF) in coir production if they are planning to reduce water or nutrient input.
- Large growers should consider conducting their own trials to assess the benefit of using AMF, particularly in relation to water and nutrient input.
- NIAB EMR will be more than happy to assist growers in trialling AMF.
- Currently, there is an on-going Innovate UK project relating to the use of AMF in substrate strawberry, which may greatly influence the commercial future of AMF in substrate strawberry production. This project will finish in October 2017.

SCIENCE SECTION

1 Introduction

Verticillium wilt of strawberry caused by *Verticillium dahliae* was traditionally controlled in field soils through the use of soil fumigants to reduce quantities of the pathogen to levels which are not economically damaging to subsequent crops. With the gradual reduction in available soil fumigants over recent years, extensive effort has gone into finding alternative control methods including the incorporation of green manures that release volatile fungitoxic compounds (so-called biofumigation), which has shown promise as a component of a disease management strategy.

In a recent Defra Horticulture LINK project (HL0177, SF 77), the incorporation of lavender waste in soils prior to planting effectively reduced Verticillium wilt severity on strawberry. Three key terpenoids were identified as being responsible for the observed suppressive effect. In a subsequent TSB funded project led by EMR, palletised lavender waste and microencapsulated terpenoids are being assessed for their efficacy in controlling *V. dahliae.* However, it is almost certain that additional control measures will still be needed. Soil inhabiting arbuscular mycorrhizal fungi (AMF) have been shown to confer a number of benefits to their host plant including enhanced pathogen resistance, so there is merit in investigating this further to ascertain if it offers potential benefits to soil grown strawberry production. In a recently completed project (funded by Interreg), NIAB EMR researchers showed that AMF can increase strawberry yield by up to 10-15% in coir, particularly when plants were subjected either to reduced nutrients or reduced water input (Robinson Boyer et al. 2016).

This project is investigating if pre-colonising strawberry plants with AMF leads to reduced incidence or severity of Verticillium wilt. It is being funded through the AHDB Horticulture studentship scheme, which supports the development of new scientists working in horticultural research. The student leading this project is Benjamin Langendorf at East Malling Research. During the project period, it has been decided, in consultation with the industry that disease tolerance work should focus on *Phytophthora* instead of wilt because most strawberry production is now on substrate instead of soil.

We have carried out the following research work to investigate: (1) whether AMF could colonise tipping plants under the weaning (humid misting) conditions, (2) whether there are interaction between AMF strains and strawberry cultivars in terms of AMF colonisation, (3) whether AMF in colonised roots of tray plants could survive a prolonged period of cold storage, (4) whether AMF colonised plants could increase tolerance to wilt, (5) whether AMF could increase plant tolerance to Phytophthora diseases, and (6) whether we could establish an *in vitro* system to study the strawberry-AMF-pathogen interaction.

Since this project is a PhD project, the presentation follows a conventional PhD thesis format: each chapter is for one specific topic. Furthermore, it should be noted that the PhD student is going to submit the thesis in early 2017 (indeed, it is very rare that PhD thesis is submitted within three years.

2 General methods

2.1 Arbuscular mycorrhiza fungi (AMF) inoculum

2.1.1 Inoculum source and inoculation

Pure cultures of five arbuscular mycorrhizal fungi (AMF) species (*Funneliformis mosseae* [T.H. Nicolson & Gerd.] C. Walker & A. Schuessler 2010, *Rhizophagus irregularis* [N.C. Schenck & G.S. Sm.] C. Walker & A. Schuessler 2010, *Claroideoglomus claroideum irregularis* [N.C. Schenck & G.S. Sm.] C. Walker & A. Schuessler 2010, *F. geosporus* [T.H. Nicolson & Gerd.] C. Walker & A. Schuessler 2010 and *Glomus microaggregatum* Koske, Gemma & P.D. Olexia 1986), were initially obtained from Plantworks Ltd, Kent, UK, as attapulgite clay/ pumice/ zeolite mix containing spores, mycelium, and colonised host plants root fragments of single AMF species. AMF inocula were always incorporated as a powder layer comprising 10 % (v/v) and applied ca. 1 cm below the surface of the potting substrate before transplantation of plantlets.

2.1.2 Most probable number (MPN) bioassay

A most probable number (MPN) bioassay was undertaken to determine the infectivity and estimate the number of propagules in each inoculum sample (Alexander, 1965; Cochran, 1950). Samples were diluted to 1/10, 1/100 and 1/1000 using autoclaved (two cycles at 121 °C for 20 min with 4 d between cycles) attapulgite clay substrate (AgSorb®, Oil-dri Ltd, Cambridgeshire, UK). As follows: one volume of the final inoculum was thoroughly mixed with 9 volumes of the diluting substrate to give a 1/10 dilution; one part of this mixture was then mixed with 9 parts of the substrate to give the 1/100 dilution and finally diluted again to give the 1/1000 dilution. For this bioassay, maize (*Zea mays* L.) was used as the trap plant, and five replicate pots each planted with three maize seeds were set-up at each dilution. The pots were then placed either in a glasshouse (temperature 20-23 °C, light:dark 16 h/8 h, additional lighting in the form of 400 W halogen) or a growth room (day and night 21-22 °C, ca. 72 % RH, light:dark 16 h/8 h, photosynthetically active radiation (PAR) of ca. 40 µmol m^{-2} s^{-1}) and the plants allowed to develop. Plants were watered as required with tap water and roots were harvested after c. six weeks. The maize roots harvested from each pot were then stained with trypan blue (see Section 2.3.1 below) and assessed microscopically for the presence of AMF structures. Based on the incidence of microscopic presence of AMF structures in the sampled roots, MPN was then estimated, using MPN tables (Cochran, 1950).

2.2 Plant materials

2.2.1 Production of micro-propagated strawberry plants

To be certain of the absence of pre-existing mycorrhizal colonisation, micro-propagated plants (hereafter named microplants) were used in several experiments. Microplants of *Fragaria* x *ananassa* cv. 'Calypso' and of *F. vesca* var. *alpina* were purchased from Hargreaves Plants Ltd, Norfolk, UK, whilst microplants of *F.* x *ananassa* cv. 'Vibrant', 'Red glory' and accession 'EM1996' and of *F. vesca* clone VSI were provided by EMR tissue culture laboratory, Kent, UK. Microplants were all established for at least two months on Murashige & Skoog (M&S) medium (Murashige & Skoog, 1962) supplemented with 0.75 % agar, 3 % sucrose, and 1.2 mL L^{-1} GA_3 and 8 mL L^{-1} IBA (a phyto-hormone) to induce rooting. *In vitro* plants were incubated in a growth room (21 °C, ~50 % relative humidity (RH), light:dark 16 h/ 8 h PAR of 40 µmol m^{-2} s^{-1}) until the plant roots had developed sufficiently to be transplanted.

2.2.2 Production of runner-tips

Pre-established strawberry mother plants (cv. 'Elsanta', 'Malling Centenary', 'Vibrant', 'Red Glory') were grown in coir bags (Botanicoir Ltd, London, UK) in a poly-tunnel or a glasshouse compartment at EMR. Runner-tips were produced within three months either under greenhouse conditions (in winter; temperature 20-23 °C, light:dark 16 h/8 h, additional lightning in the form of 400 W halogen bulbs was used with ample irrigation, appropriate fertilisation regimes and pest control) or poly-tunnel conditions (in spring/ summer; natural light and temperatures, ample irrigation, appropriate fertilisation regimes and pest control). Inflorescences emerging from the mother plants were removed regularly to stimulate runner production. Further runner-tips of cv. 'Vibrant' were obtained from R W Walpole Ltd, Norfolk, UK), and of cv. 'Red glory' from Edward Vinson Plants Ltd, Kent, UK). Once runner plantlets contained at least three compound leaves they were cut away from the mother plants and used for experiments (weaning and AMF inoculation).

2.2.3 Growth medium and strawberry plantlet weaning

The roots of the microplants were washed with purified water to remove any adhering agar and nutrients. Microplants were then gently transplanted individually in each cell of plastic trays (40 cells, ~46 cm^3 per cell, B&Q 40 Cell Insert 08535B, Kent, UK; or 56 cells, 70 cm^3 per cell, Agrii Ltd, Kent, UK) that were filled up with non-autoclaved coir (Botanicoir Ltd, London, UK) or autoclaved (two cycles at 121 °C for 20 min with 4 d between cycles) vermiculite medium (Sinclair horticulture Ltd, Lincoln, UK) fertilised with 0.25 g L^{-1} of autoclaved (one cycle at 121 °C, 20 min) bone-meal, a complex N and P source to encourage mycorrhizal development (3.5 % N, 7.4 % P; Verve, Hampshire, UK). Plantlets were then weaned in plastic propagator units with transparent, vented lids (52 × 42.5 × 24 cm, Stewart Plastics Ltd, Oxon, UK) kept in a growth room (Meridian Refrigeration Ltd, Croydon, UK; day and night 21-22 °C, ~72 % RH,

light:dark 16 h/8 h, PAR of ~40 µmol m^{-2} s^{-1}) (Figure 2.1). Both adjustable vents present on the lid of the propagator were kept closed initially (1 week), and then left open (1 week) before the lid was completely removed. Each plantlet was then watered as required with 10 mL of purified water and no additional fertiliser was added.

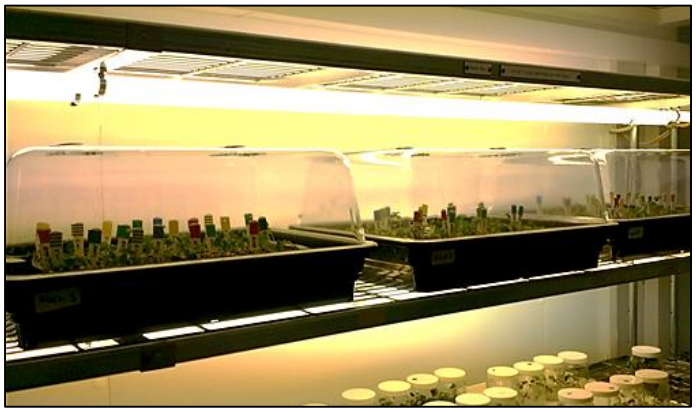

Figure 2.1. Microplants weaned inside plant propagators that were kept under growth room conditions.

Freshly cut runner tips were immediately pinned-down in standard plastic module trays (56 cells, 70 cm^3 per cell, Agrii Ltd, Kent, UK; or 48 cells, 70 cm^3 per cell, Desch Plantpak Ltd, Essex, UK) filled up with the potting mix consisting of 7 parts Irish dark peat (Clover Peat Products Ltd, Dungannon, Ireland) and 3 parts 2.0-5.0 mm perlite (Sinclair Horticulture Ltd, Lincoln, UK) (Figure 2.2A). The potting mix was limed with 16 g L^{-1} of non-autoclaved dolomite lime (Omya UK Ltd, Derbyshire, UK) to give a pH of ca. 7 and fertilised with 0.25 g L^{-1} of autoclaved (one cycle at 121 °C, 20 min) bone-meal (Verve, Hampshire, UK). Immediately after transplantation in plastic trays, runner-tips were placed in a misting cabinet (daily mean temperature: > ~20 °C, no artificial light, daily mean RH > 90 %) and intermittently sprayed with tap water using a Macpenny Solarmist VTL misting system (Wright Rain Ltd, Hampshire, UK) for at least 15 d (misting of ca. five seconds at frequencies, depending on light conditions, ranging from six minute intervals on bright days to 20 minutes intervals on dull days according to the manufacturer guidelines) (Figure 2.2B). The plant propagation was carried out in a glasshouse compartment (ca. 19 °C, daily mean RH 72 %, 16 h/8 h light:dark cycle with additional lighting supplied in the form of 400 W halogen bulbs) (Figure 2.2C). The plug plants were watered once a day with tap water. No additional fertiliser was added.

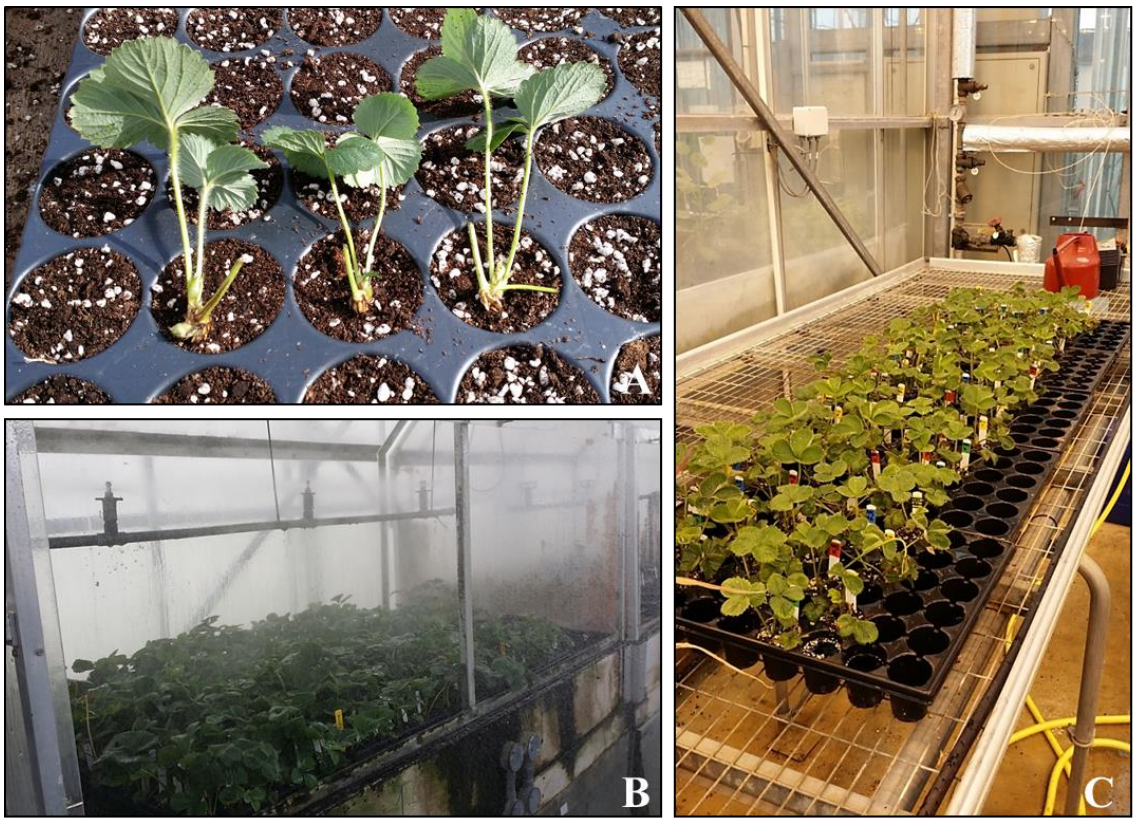

Figure 2.2. (A) Runner-tips ready to be pinned down on Irish dark peat/perlite mix (7:3, v/v). (B) Strawberry plug transplants inside misting system cabinet. (C) Plug plants growing under glasshouse condition at 7 weeks post transplantation.

2.3 Arbuscular mycorrhiza fungi quantification

2.3.1 Root sampling and staining

To observe AMF structures within the strawberry roots, samples were randomly picked and cleaned with tap water to remove substrate particles. The roots were then cleared in 2 % (w/v) potassium hydroxide solution (KOH) for 1 h at 90 °C. Cassettes containing roots were then rinsed three times with tap water before being submerged for 30 min in 2 % (v/v) hydrochloric acid (HCl) at room temperature. The HCl was then discarded and the roots samples covered with 0.05 % (w/v) trypan blue in lactoglycerol (lactic acid, glycerol, water – 1:1:1 as Kormanik & McGraw, 1982 but omitting phenol) for 1 h at 90 °C in a water bath. After de-staining in 50 % (v/v) glycerol-water, randomly selected root segments (30 per sample) were permanently mounted (using polyvinyl alcohol lactoglycerol as described in www.invam.wvu.edu/methods/recipes) on two different slides (15 root fragments per slide) and a cover-slip was carefully positioned on each slide. Root cells were gently separated by applying slight pressure to the root.

2.3.2 Root length colonisation (RLC) assessment

To quantify total root length colonised by AMF in the sample, the grid-line intersect method of McGonigle *et al*. (1990) was used. For each root sample, AMF colonisation was assessed on 100 intersects of root tissue and expressed as a percentage of root length colonisation. Slides were examined under a Leitz Diaplan microscope with magnifications at X250. Sections of root were recorded as either positive or negative for any mycorrhizal structures as they crossed an intersect line of an eyepiece gratitude (McGonigle et al. 1990).

3 AMF Colonisation under misting and survival during cold storage

3.1 Introduction

Pre-inoculation of horticultural crops with arbuscular mycorrhizal fungi (AMF) before transplanting has been proposed as an environmentally-friendly method to promote plant growth and health by protecting crops against biotic and abiotic stresses (Varma and Schüepp 1994, Corkidi et al. 2004, Vestberg et al. 2004a, Rouphael et al. 2015). Strawberry (*Fragaria* x *ananassa*) production systems make AMF pre-inoculation at the weaning stage relatively straightforward for both *in vitro* and runner derived plantlets. AMF can colonise strawberry roots in different types of substrates and growing conditions (Holevas 1966, Daft and Okusanya 1973, Robertson et al. 1988, Hršelová et al. 1989, Vestberg 1992a, Williams et al. 1992, de Silva et al. 1996). However, similar studies have not been carried out with runner-tip derived strawberry plant during the weaning stage.

The substrates commonly used to propagate strawberry plantlets deriving from *in vitro* and runner-tips are coir, peat, perlite and vermiculite (Vestberg et al. 2000, D'Anna et al. 2002, Corkidi et al. 2004, Rouphael et al. 2015, Treder et al. 2015), but AMF propagules are not usually present in these growing media (Azcón-Aguilar and Barea 1997). AMF inoculation of substrates containing peat mixed with sand, perlite, zeolite and/or vermiculite has been demonstrated to result in successful AMF colonisation of strawberry plantlets (Vosatka et al. 1992, Williams et al. 1992). In contrast, other studies have reported that certain types of peat had negative effects on AMF root colonisation of strawberry *in-vitro* derived plantlets during propagation (Niemi and Vestberg 1992, Vestberg et al. 2000, Corkidi et al. 2004, Palencia et al. 2013). Therefore, it remains unclear whether the negative effect of peat on AMF colonisation was due to high input of fertilisers (e.g. phosphorus) or the results of other chemical and biological properties of the peat itself (Martinez et al. 2013, Palencia et al. 2013). As the majority of strawberry runner-tips are rooted on peat-based media (Durner et al. 2002), tests are required to verify whether or not strawberry runner-tips can be pre-colonised by AMF under such conditions. Moreover, plant propagators or misting systems are required to maintain damp condition for at least two weeks to ensure plant acclimatisation and rooting (Durner et al. 2002, Treder et al. 2015). Some evidence suggests that AMF root colonisation may also be limited under wet conditions as a result of lower oxygen availability reducing AMF propagules survival (Thormann et al. 1999, Miller 2000).

Differences in AMF root colonisation levels among different strawberry cultivars has previously been documented under field (Robertson et al. 1988) and glasshouse (Chávez and Ferrera-Cerrato 1990, Vestberg 1992c) conditions, while other studies have found no variation in AMF root colonisation levels among strawberry cultivars grown under glasshouse conditions (Robertson et al. 1988, Cekic and Yilmaz 2011). Furthermore, the positive effect of AMF

inoculation on strawberry plant growth is controversial. Several studies demonstrated the beneficial effects of AMF inoculation on strawberry growth during propagation (Kiernan et al. 1984, Chavez and Ferrera-Cerrato 1987, Hršelová et al. 1989, Niemi and Vestberg 1992, Vestberg 1992c, Vestberg et al. 2000, Borkowska 2002, Stewart et al. 2005, Castellanos-Morales et al. 2010, Fan et al. 2011, Boyer et al. 2014), whilst others reported either limited (Cekic and Yilmaz 2011, Garland et al. 2011, Palencia et al. 2015) or negative effects (Chávez and Ferrera-Cerrato 1990, Hršelová et al. 1990, Vestberg et al. 2004a).

Before transplantation strawberry plugs need to be cold stored at -2 °C for various lengths of time in order to achieve sufficient chilling and to schedule cropping (Lieten et al. 2005). The potential consequences of a prolonged cold storage at freezing temperatures on the survival and infectivity of AMF propagules (i.e. spores, colonised roots and extraradical hyphae) in the root ball of strawberry plugs are unknown; although several studies suggest that AMF propagules of *Glomus* species have the ability to endure cold, including winter freezing conditions (Safir et al. 1990, Addy et al. 1994, Addy et al. 1997, Kabir et al. 1997, Addy et al. 1998, Klironomos et al. 2001, Juge et al. 2002).

Four experiments were conducted to investigate whether inoculation with AMF during weaning of ex-tissue culture (hereafter named microplants) and runner-tip derived strawberry plantlets of different cultivars could result in well-colonised plug transplants. Specific objectives included determining: (1) whether AMF can colonise roots of strawberry plantlets under damp condition in soil-less substrate; (2) whether inoculated AMF species differ in their ability to colonise popular strawberry cultivars; (3) whether AMF species differ in their capacity to colonise roots of different plant sizes; (4) whether early AMF colonisation increases growth of strawberry plugs during the weaning and subsequent post-transplanting growth. Finally, one experiment was carried out to study the freezing tolerance (i.e. tolerance to the formation of ice in the strawberry root ball for several months) of AMF colonising strawberry plug roots.

3.2 Materials and methods

A total of five experiments were carried out in 2014-2015; the duration of each experiment varied from six to 45 weeks. A schematic representation of the experiment setup is shown in Figure 3.1 (colonisation during the weaning stage) and Figure 3.2 (AMF survival during cold storage), whilst Table 3.1 gives the summary of experimental details.

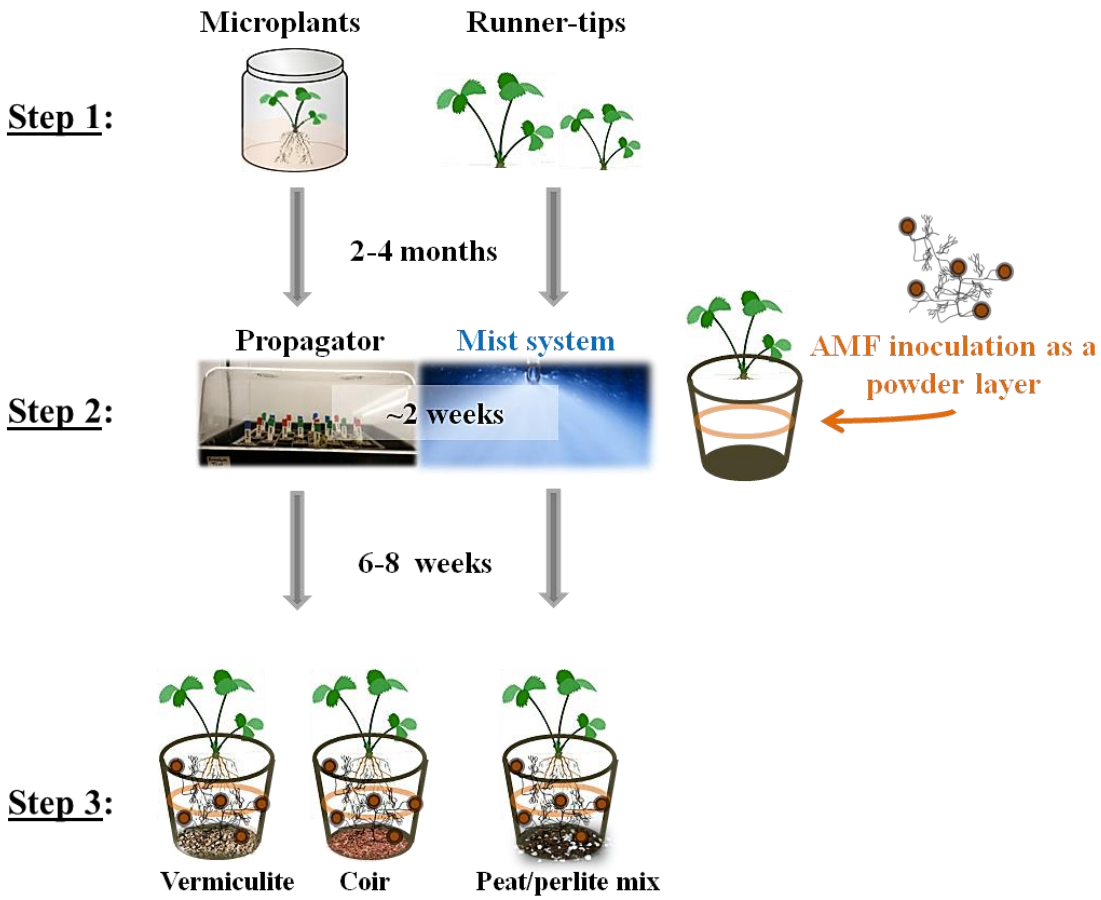

Figure 3.1. Schematic representation of the experimental setup to study the effect of arbuscular mycorrhiza fungi (AMF) pre-inoculation of strawberry transplants during weaning and propagation. Microplants and runner-tips were produced (Step 1). Planting materials were pinned down in vermiculite, coir, or Irish dark peat/perlite mix (7:3, v/v) and then weaned for ~2 weeks inside a propagator or misting cabinet (Step2). Newly formed roots were assessed for the extent of AMF colonisation at the end of the plug transplant propagation (Step 3)

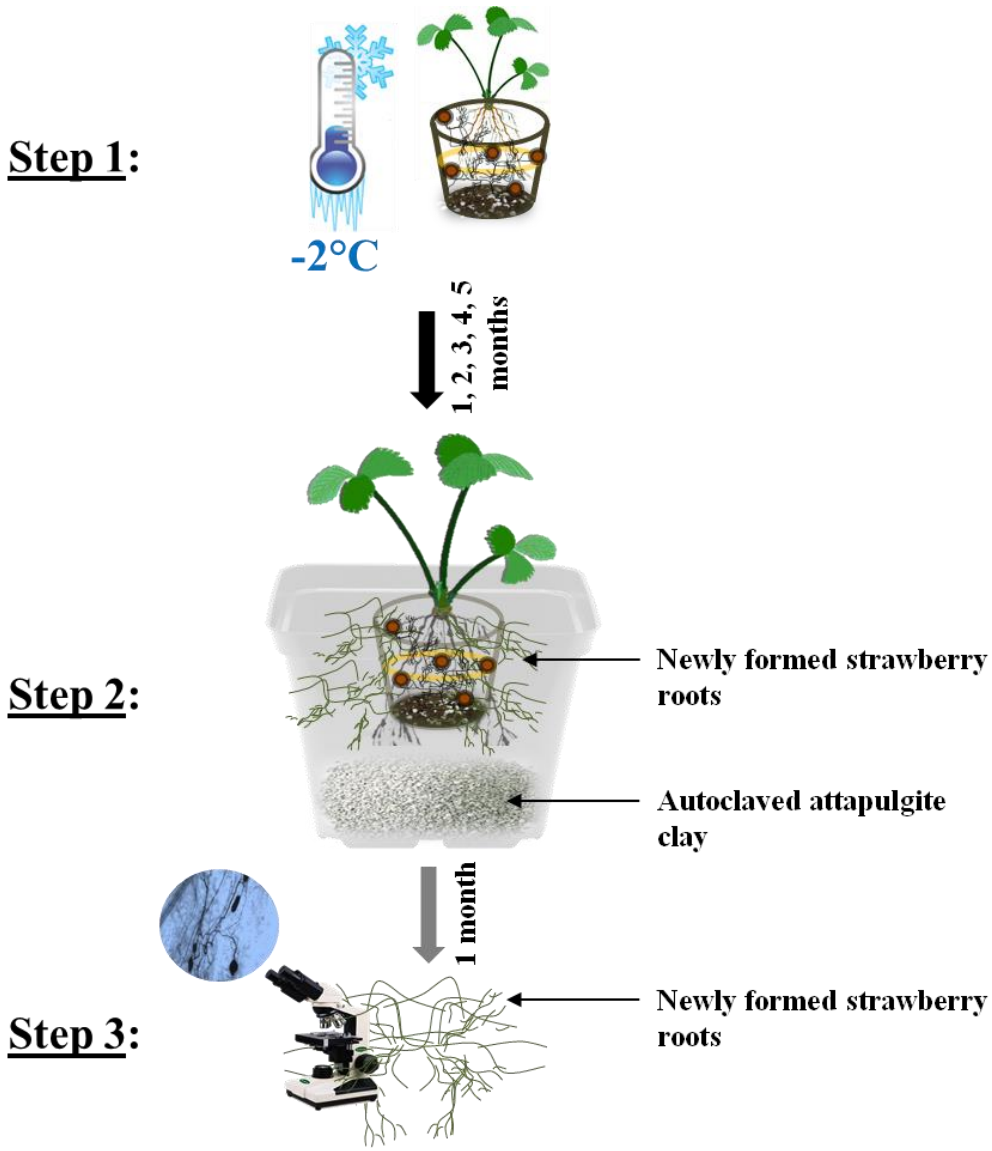

Figure 3.2. Schematic representation of the experimental setup to study the effect of cold storage on the survival of arbuscular mycorrhizal fungi (AMF) in colonised strawberry roots. Cold storage at -2 °C of AMF pre-inoculated strawberry plug transplant (Step 1). Plants were re-potted at monthly intervals (1 to 5 months) in autoclaved attapulgite clay and placed in a growth room at 22 °C (Step 2). Newly formed roots were assessed for the extent of AMF colonisation one month after transplantation (Step 3).

Table 3.1. Details of experiments to study arbuscular mycorrhizal fungi (AMF) pre-inoculation of strawberry plug during the weaning stage (Experiment 1-4) and the effect of cold storage at -2 °C on the survival of AMF in strawberry roots (Experiment. 5).

	Experiment				
	1	**2**	**3**	**4**	**5**
Total no. treatments	5	4	10	48	30
AMF species and controls[a]	*F.m*, *R.i*, *C.c,* Cb^-, Cb^+	*R.i,* Cb^+	*F.m*, *R.i*, *C.c,* Cb^-, Cb^+	*F.m*, *R.i*, *C.c*, *G.m*, *F.g,* Cb^+	*F.m*, *R.i*, *C.c*
Plant cultivars[b]	EM-1996	V	RG, V	E, MC, RG, V	RG, V
Plant (runner) size categories	-	-	-	Small/Large	-
Weaning substrate[c]	Vermiculite	Vermiculite or Coir	Peat/perlite mix	Peat/perlite mix	Peat/perlite mix
Re-growth substrate	-	-	-	-	Attapulgite clay
Cold storage duration at -2 °C (months)	-	-	-	-	1, 2, 3, 4, 5
No. replicates per treatment	12	21	10	6	11
No. blocks (trays)	3	3	5	6	-
Plant material types[c]	Microplant	Microplant	Runner-tip	Runner-tip	Runner-tip
Weaning substrate autoclaved	Yes	No	No	No	No
AMF inoculum washing per plant (v/plant)	1 mL	10 mL	1.5 mL	10 mL	-
Plastic tray size (no. cells)[d]	40	56	48	48	56
Substrate fertilisation[d]	Bone-meal	-	Bone-meal	Bone-meal	Bone-meal
Dolomite lime amendment[d]	-	-	Limed	Limed	Limed
Weaning methods[d]	Propagator	Propagator	Misting system	Misting system	Misting system
Experiment location	Growth room	Growth room	Glasshouse	Glasshouse	Growth room
Start date	31/01/14	31/03/15	03/03/14	03/06/14	23/09/14
Duration of weaning + propagation (weeks)	2 + 4	2 + 6	2 + 4	2 + 5	2 + 16

[a] Abbreviations *F.m*, *R.i*, *C.c*, *G.m* and *F.g* respectively stand for the single AMF species inoculated: *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microaggregatum*, *Funneliformis geosporum*. Cb^-: a control inoculated with autoclaved attapulgite clay; Cb^+: a control inoculated with an autoclaved equal mix of AMF species used and inoculated with bacterial washing

[b] Abbreviations E, MC, RG, and V respectively stand for the strawberry cultivars: 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'

[c] See Section 2.2.1 for vermiculite or coir and Section 2.2.2 for peat/perlite mix

[d] See Section 2.2.3 for experimental details

3.2.1 Inoculation of arbuscular mycorrhiza fungi (AMF)

All AMF species used in the experiments (Table 3.1) were provided by PlantWorks Ltd, Kent, UK, and inoculated as described in Section 2.1.1. The number of infective propagules of each AMF inoculum (Table 3.2) was determined using a most probable number (MPN) bioassay procedure described in Section 2.1.2. In addition, two non-AMF controls were included: (1) Cb^- inoculated with autoclaved (two cycles at 121 °C for 20 min with 4 d between cycles) attapulgite clay (AgSorb®, Oil-dri Ltd, Cambridgeshire, UK) to assess possible physico-chemical effects of the inoculum carrier, and (2) Cb^+ inoculated with autoclaved (one cycle, 121 °C, 20 min) inoculum or an equal mix of the single AMF species used (Table 3.1). To equalise the starting microbial community, 1 mL, 1.5 mL or 10 mL of AMF inoculum washing solution was also added to each plantlet (Table 3.1). This was produced by suspending 1 g of live inoculum in 10 mL of purified water, then vortexed for ~10 min and filtered through a 45 µm sieve (Laboratory test sieve, Endecotts Ltd, London, UK).

Table 3.2. Number of arbuscular mycorrhiza fungi (AMF) infective propagules per mL of inoculum substrate carrier used to inoculate strawberry plants in experiments 1-5.

Experiment	**AMF species**	**Propagules mL^{-1}**
1, 3, 4	*Funneliformis mosseae*	> 1600
5	*Funneliformis mosseae*	170
1, 3, 4	*Rhizophagus irregularis*	> 1600
2	*Rhizophagus irregularis*	70
5	*Rhizophagus irregularis*	170
1, 3, 4	*Claroideoglomus claroideum*	> 1600
5	*Claroideoglomus claroideum*	23
4	*Glomus microagregatum*	79
4	*Funneliformis geosporum*	350

3.2.2 Source of plant materials, weaning and propagation

Either microplants or runner-tips (Table 3.1) were used to mimic different propagation systems. A randomised block design was used in all the five experiments except for Experiment 5 (see Section 3.2.4 below). In Experiment 4, there were also two plant crown size groups named grade ‘Large’ and ‘Small’ (Table 3.3). Experiments were all carried out in plastic trays that were either filled up with vermiculite, coir or Irish dark peat/ perlite mix (Table 3.1). The background nutrient status of the weaning substrates were analysed before plant transplantation (Table 3.4). The microplants or runner-tips were transplanted and weaned as described in Section 2.2.3. In Experiment 1 and 2, each plantlet was watered as required with 10 mL of purified

water, while in the other three experiments plants were watered once a day with tap water. No additional fertiliser was added. However, the absence of fertilisation resulted in rapid occurrence of nutrient deficiency symptoms in Experiment 1 (Figure 3.4). Thus, each plant received with 10 mL of half-strength Rorison's nutrient solution minus phosphate, three times a week (Hewitt and Bureaux 1966), which rapidly alleviated the nutrient deficiency symptoms. During the growing period of Experiment 2, the plants were infested by spider-mites, which were controlled by weekly release of spider-mite predators (*Phytoseiulus persimilis*; Phytoline p; Syngenta Bioline Ltd, Essex, UK) until the end of the experiment. In Experiment 5, after weaning plants were first grown for 48 d under glasshouse conditions (see Section 2.2.3) before their transfer for 51 d to a poly-tunnel with natural shorter days and cooler temperatures to induce dormancy (Figure 3.7a). Plants were continued to be watered daily with tap water. At the end of the propagation period, plants were then acclimated to lower temperatures for 24 d in a dark compartment cooled to 2 °C. Watering ceased from this point onwards. Finally, strawberry plugs were cold stored at -2 °C for different periods of time (Figure 3.8B; see Section 3.2.4 below).

Table 3.3. Average strawberry runner crown diameter for the explant size of 'large' and 'small' in experiment 4.

Strawberry cultivar	**Crown diameter (mm)***	
	'Large'	**'Small'**
'Elsanta'	8.9 ± 0.4	5.2 ± 0.3
'Malling Centenary'	6.9 ± 0.4	4.4 ± 0.2
'Red Glory'	5.9 ± 0.2	4.1 ± 0.2
'Vibrant'	7.8 ± 0.3	4.4 ± 0.1

*mean ± SE, *n* = 21

Table 3.4. Results of background nutrient status[a] of the growing media used in the various experiments.

Experiment	Medium	pH	NO_3^-	NH_4^+	P	K	Mg	Ca^{2+}
			mg kg^{-1}					
1 & 2	Vermiculite	8.1	< 1.2	29.8	< 1.2	36.2	21.9	1.2
2	Coir	6.6	< 3.7	19.8	< 6.2	30.9	< 1.2	1.9
3	Irish dark peat/perlite mix	7.0	13.9	63.1	2.1	8.8	40.2	25.1
4	Irish dark peat/perlite mix	7.2	39.9	35.2	2.5	6.2	58.4	37.7
5	Attapulgite clay	6.4	< 0.7	18.8	3.2	81.0	117.6	142.9

[a]Growth medium analysis was determined by NRM Laboratories, Berkshire, UK. NO_3^- was determined by ion chromatography and NH_4^+ by colorimetric analysis. P, K, Mg and Ca was analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy). Only the substrate attapulgite clay used for plant re-growth was analysed. Hence, the Irish dark peat/ perlite mix used for plant weaning in Experiment 5 was not analysed for background nutrient status.

3.2.4 Re-growth of strawberry plugs after freezing cold storage

In Experiment 5, strawberry plants stored at -2 °C were moved out of the cold store, re-potted into 250 mL plastic pots (7 × 7 × 8 cm, Desch Plantpak Ltd, Essex, UK) and placed to a growth room after 1, 2, 3, 4 or 5 months in the cold store. The dead leaves were cut-off and the attapulgite clay substrate (AgSorb®, Oil-dri Ltd, Cambridgeshire, UK) was autoclaved (two cycles at 121 °C for 20 min with 4 d between cycles). A square piece of filter paper was placed inside each pot to avoid substrate leakage during watering. A randomised design was used for each of the five storage periods. Plants were grown for one month in the growth room (Meridian Refrigeration Ltd, Croydon, UK; day and night 21-22 °C, light: dark 16 h/8 h; photo synthetically active radiation (PAR) of 37-42 µmol m^{-2} s^{-1} with RH ca. 70 %; Figure 3.8C) and watered from the bottom twice a week with 4 L of purified water. No additional fertiliser was added.

3.2.5 Root sample analysis and plant growth

With the exception of Experiments 2 and 5, plants were non-destructively sampled at the end of each experiment to enable AMF root colonisation and plant growth parameters to be assessed. For all experiments, fresh roots were sampled at the end of the experimental period (with an additional root sampling before cold storage in Experiment 5) and stained to assess

endophyte (Section 2.3.2) and AMF (Section 2.3.1) colonisation. Aseptate inter- or intracellular linear hyphae associated with vesicle and/or arbuscule structures were characterised as AMF colonisation. Microsclerotium like structures, moniliform group of fungal cells and non-linear hyphae not associated with vesicles or arbuscules were recorded as dark septate endophytes (DSE) colonisation.

Plant crown size was measured by a calliper measurement at the widest point as was plant height by a calliper measurement of the highest leaf stalk, measured from the crown to the tip of the stalk; Experiment 1 and 3) and plant survival (Experiment 1-4). To measure the total plant fresh weight (Experiment 1 and 2), plants were harvested and roots were then gently washed with tap water to remove substrates particles and then weighted. For the dry weight (Experiment 2), the same procedure was followed; plants were oven dried at 80 °C for 3 d before weighing. To calculate the root weight ratio (RWR: root dry weight as a fraction of total plant dry weight), roots were weighted after being separated from the shoot. Strawberry plugs that were non-destructively harvested in Experiment 1, 3 and 4 were used in other experiments (See Chapter 5).

3.2.6 Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). AMF root length colonisation (% RLC) was analysed by ANOVA after arcsine square root transformation to satisfy normality. In Experiment 1, treatment effects were tested using a one-way ANOVA while a two-way ANOVA was used to test for treatment effects in Experiment 2 and 3. In Experiment 4, treatment effects were analysed using an unbalanced three-way ANOVA. Only three of the six blocks were sampled for % RLC analysis in Experiment 4 because of time constraints. In Experiment 5, the relationship between % RLC and percentage of DSE root colonisation (% DSE) was analysed using a linear regression. In Experiment 5, the effect of cold storage duration on % RLC was assessed using a three-way ANOVA with storage time (1 to 5 months), AMF treatment (single AMF species inoculated: *F. mosseae* or *R. irregularis* or *C. claroideum*) and strawberry cultivar ('Vibrant' or 'Red Glory') as factors. The main objective of this analysis was to test the effect of cold storage duration on AMF survival in colonised roots. However, it was suspected that plant roots with high DSE colonisation were more likely to have lower AMF root colonisation due to competition for space or other indirect effects of DSE. Therefore, DSE root colonisation level was included as a covariate in the ANOVA to remove its influence on AMF root colonisation. The significant differences among individual treatments were determined using a least significant difference (LSD) post-hoc test. Additionally, AMF survival in AMF inoculated and non-inoculated plants in Experiment 4 was compared using a generalised linear model (GLM) with residual errors assumed to follow binomial distributions; the logit link function was used.

Only significant differences are reported in the text. For the plant growth parameter data, if there was no significant difference among the AMF species, then the data were pooled and treated as single AMF group effect. The non-mycorrhizal (NM) control (Cb^+ and Cb^-) data were treated similarly.

3.3 Results

3.3.1 Influence of weaning conditions on AMF colonisation

Microscopic assessments of strawberry plug roots showed the presence of fungal structures for all the plants tested irrespective of the type of plant material, growth substrate, plant size and cultivar. The presence of arbuscules and/or vesicles confirmed the presence of AMF (Figure 3.3-3.5; 3.7; 3.8a-f). The occurrences of microsclerotium-like structures and/or moniliform group of fungal cells were sporadically observed in the root cortex of plants in Experiment 4, while they were more abundant in Experiment 5, confirming the presence of DSE (Figure 3.6; 3.8a-f).

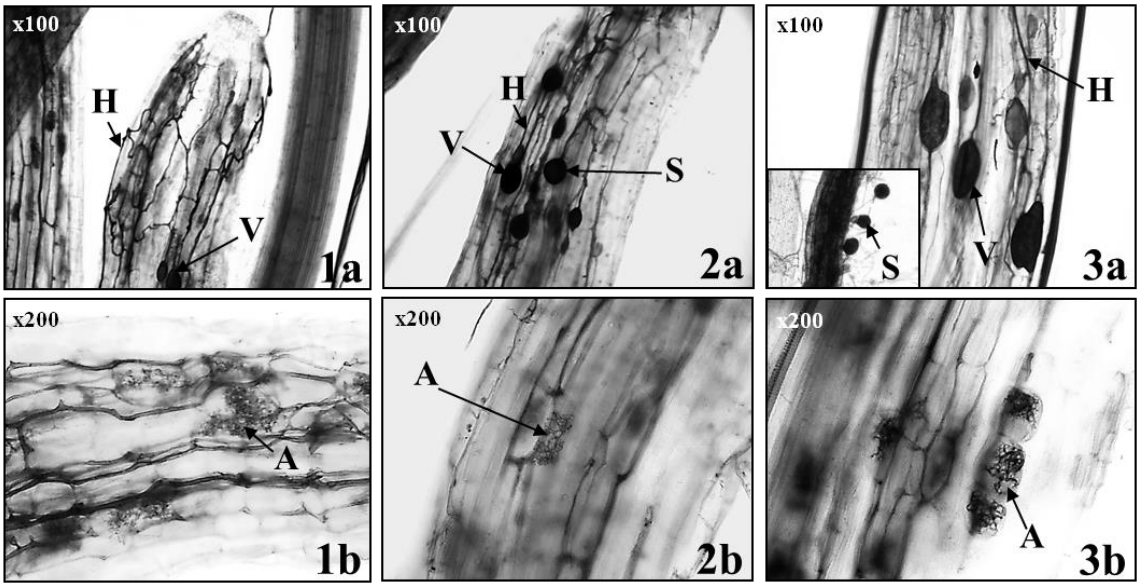

Figure 3.3. Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug ('EM-1996') after 6 weeks cultivation in autoclaved vermiculite in a growth room at 22 °C (Experiment 1). The numbers represent the AMF species: (1) *Funneliformis mosseae*; (2) *Rhizophagus irregularis*; (3) *Claroideoglomus claroideum* while the adjoining letters represent (a) Various mycorrhizal structures (a) or Arbuscules (b). Letters next to black arrows are A: arbuscule, V: vesicle, H: hypha, S: spore. The scale bar feature was not available in the camera used for image acquisition thus the magnification is reported instead.

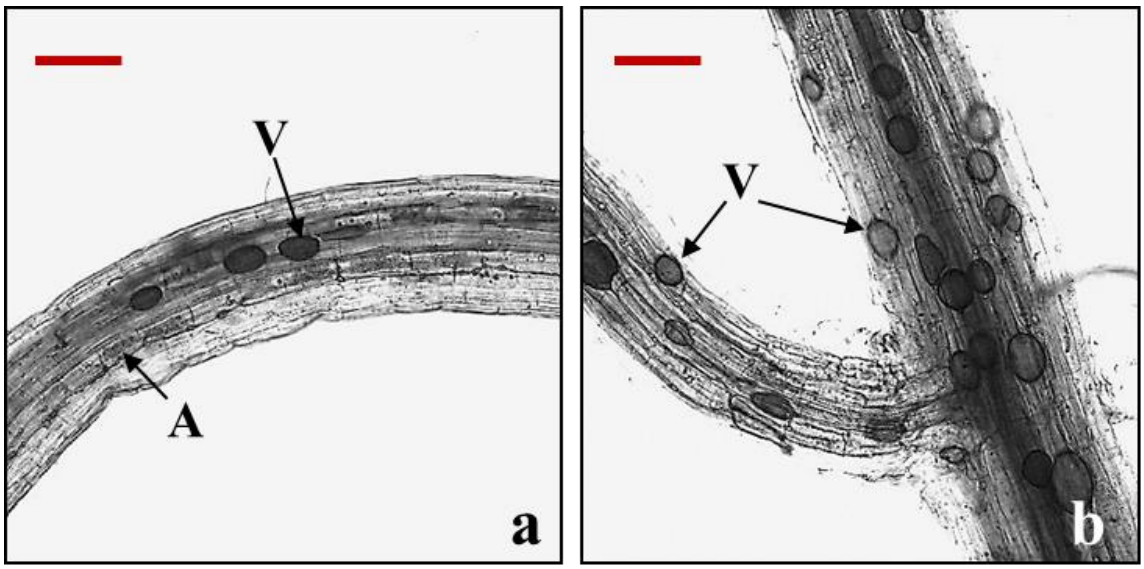

Figure 3.4. Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug cv. 'Vibrant' after 8 weeks cultivation in (a) coir and (b) vermiculite in a growth chamber at 22 °C (Experiment 2). Longitudinal squash of roots stained with trypan blue colonisation by single AMF species *Rhizophagus irregularis*. Letters next to black arrows are A: arbuscule and V: vesicle (red scale bars represent 100 µm).

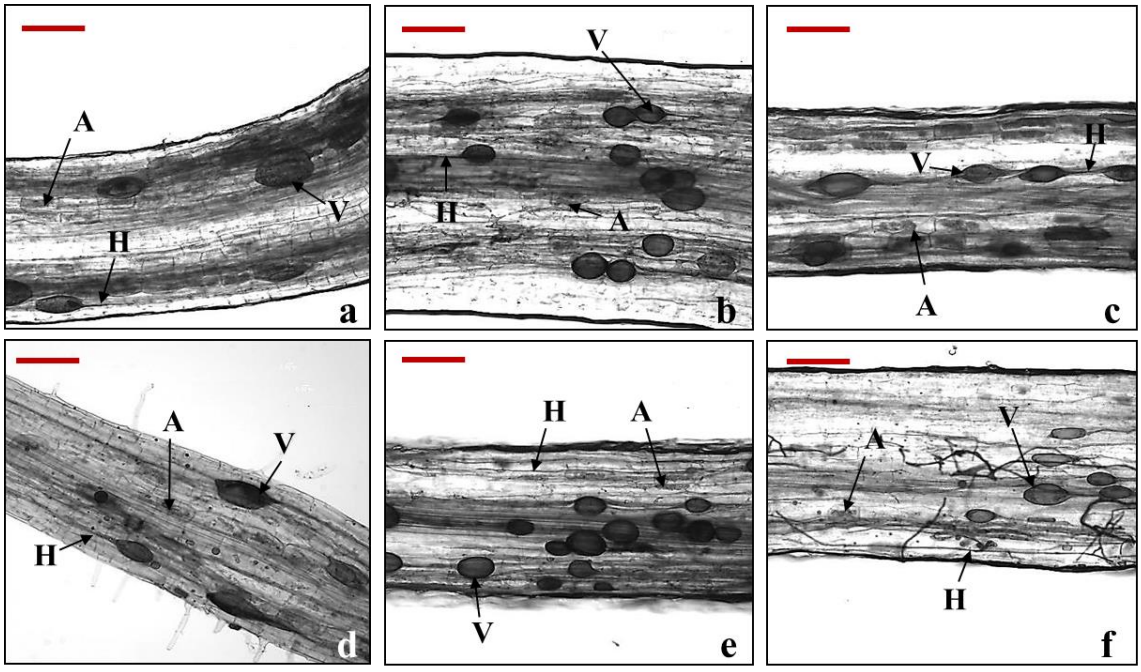

Figure 3.5. Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug cv. 'Vibrant' (a-c) and 'Red Glory' (d-f) after 6 weeks of cultivation in the Irish peat/perlite mix (7:3; v/v) under glasshouse conditions (Experiment 3). Longitudinal squash of roots stained with trypan blue colonisation by single AMF species: (a, d) *Funneliformis mosseae*; (b, e) *Rhizophagus irregularis*; (c, f) *Claroideoglomus claroideum*. Letters next to black arrows are A: arbuscule, V: vesicle, H: hypha (red scale bars represent 100 µm).

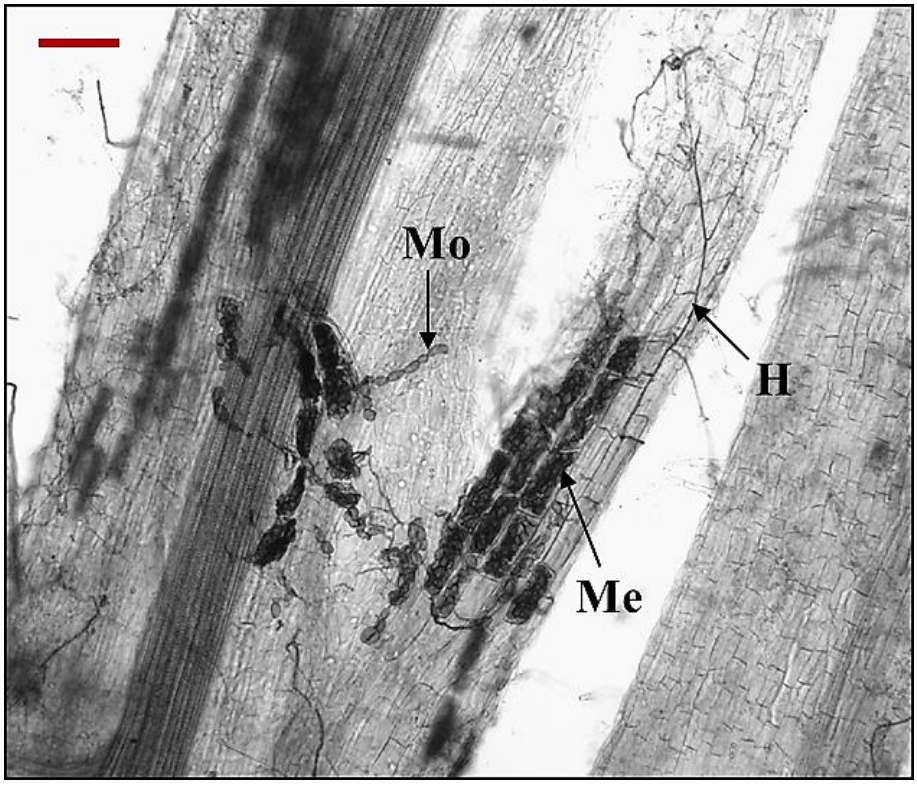

Figure 3.6. Root colonisation by dark septate endophytes (DSE) of *Fragaria* x *ananassa* of cv. 'Elsanta' after 7 weeks of cultivation in the Irish dark peat/perlite mix (7:3; v/v) under glasshouse conditions (Experiment 4). Letters next to black arrows are H: hypha, Mo: moniliform cell and Me: microsclerotium (red scale bars represent 100 µm).

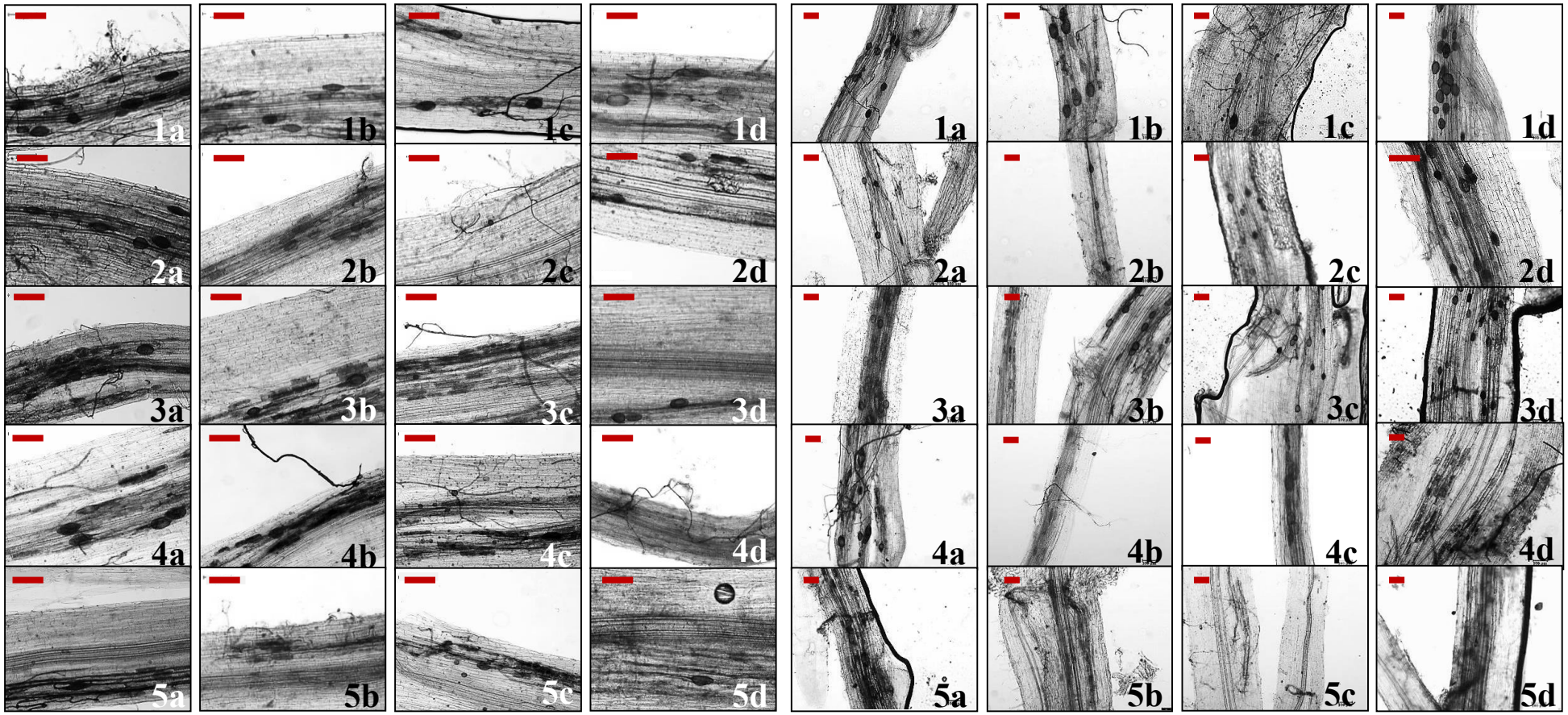

Figure 3.7. Root colonisation by arbuscular mycorrhiza fungi (AMF) of two different strawberry plant sizes ('Large'-left picture block; 'Small'-right picture block) after 7 weeks in the Irish peat/perlite mix (7:3; v/v) under glasshouse conditions (Experiment 4). Letters represent plants cultivars (a) 'Elsanta', (b) 'Malling Centenary', (c) 'Red Glory', (d) 'Vibrant'. Longitudinal squash of roots stained with trypan blue colonisation by single AMF species: (1) *Funneliformis mosseae*; (2) *Glomus microagregatum*; (3) *Rhizophagus irregularis*; (4) *Claroideoglomus claroideum*; (5) *Funneliformis geosporum* (red scale bars represent 100 µm).

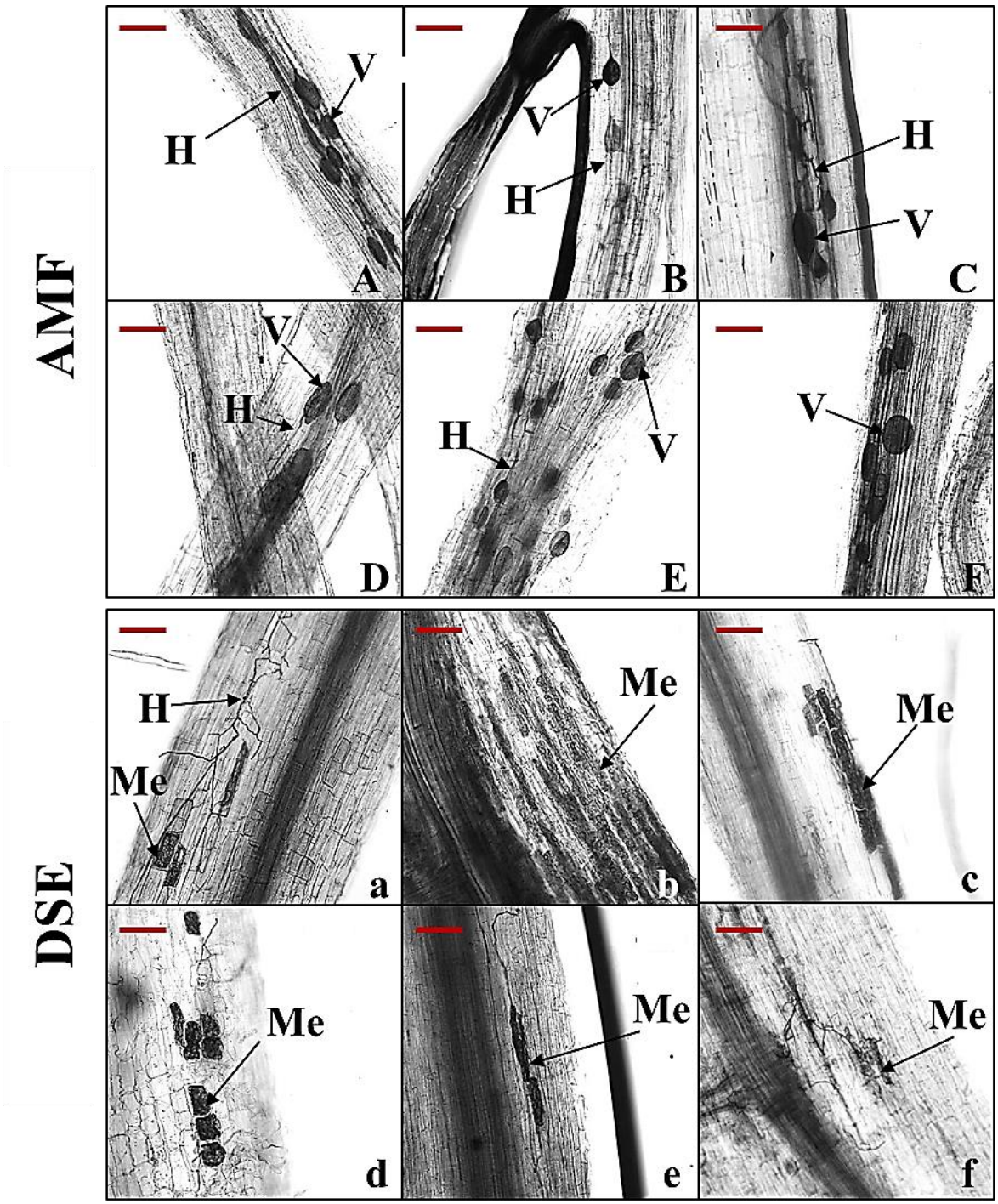

Figure 3.8. Root colonisation by arbuscular mycorrhiza fungi (AMF: A-F) or by dark septate endophytes (DSE: a-f) of *Fragaria* x *ananassa* plug of cv. 'Vibrant' (A-C and a-c) and 'Red Glory' (D-F and d-f) after 113 d in peat/perlite mix (7:3, v/v) under glasshouse/poly-tunnel conditions (Experiment 5). Longitudinal squash of roots stained with trypan blue inoculation with single AMF species: (A, D and a, d) *Funneliformis mosseae*; (B, E and b, e) *Rhizophagus irregularis*; (C, F and c, f) *Claroideoglomus claroideum*. Letters next to black arrows are V: vesicle, H: hypha and Me: microsclerotium (red scale bars represent 100 µm).

In Experiment 1 the use of vermiculite as a substrate proved equally optimal for *F. mosseae*, *R. irregularis* and *C. claroideum* root colonisation. Irrespective of the AMF species tested, average % RLC at 6 weeks was c. 94 % (Figure 3.9A) and there were no significant differences between the frequency of arbuscules or vesicles for the three AMF species tested (Figure 3.9B, C). In Experiment 2, vermiculite and coir were both conducive for root colonisation by *R. irregularis*. However, there was a large difference in % RLC between the two substrates (Figure 3.9D; $F_{1,14} = 54.7$; $P < 0.001$). Average % RLC for coir was 40 % and 76 % for vermiculite.

In Experiment 3, the Irish dark peat/perlite mix used for the weaning of runner-tips allowed *F. mosseae*, *R. irregularis* and *C. claroideum* to equally colonised plant roots. No significant differences were found between 'Vibrant' and 'Red Glory' cultivars. Average % RLC at 8 weeks ranged from 67 to 81 %. In addition, there were no significant interactions between AMF and cultivar (Figure 3.9E). Similarly, the frequency of arbuscules (Figure 3.9F) and vesicles (Figure 3.9G) did not depend on the AMF species inoculated. Arbuscules and vesicles frequencies were not affected by strawberry cultivar either. Finally, there were no significant interactions between AMF and cultivar for arbuscules or vesicles frequencies.

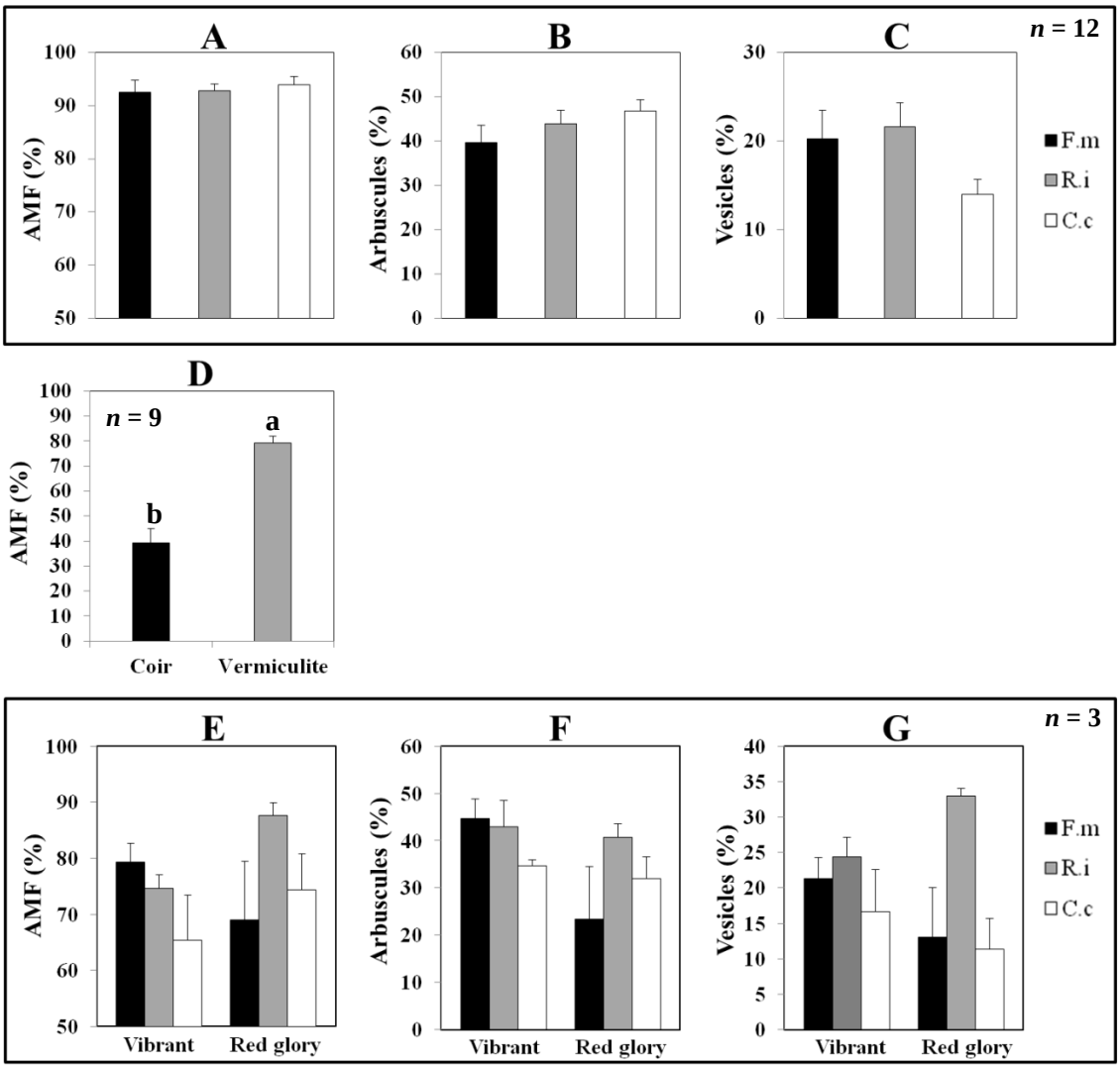

Figure 3.9. Percentage root colonisation by arbuscular mycorrhizal fungi (% RLC), arbuscules and vesicles in Experiment 1 (A-C), Experiment 2 (D) and Experiment 3 (E-G). The abbreviations F.m, R.i and C.c respectively stand for *Funneliformis mosseae*, *Rhizophagus irregularis* and *Claroideoglomus claroideum*. In Experiment 2, only *R. irregularis* was the AMF used. Root samples originated from two plants in Experiment 2 but from individual plants in all the other experiments. No AMF colonisation observed in non-mycorrhizal controls (Cb^- and/or Cb^+). Bars represent one standard error (SE), and *n* is the number of replicates per treatment.

In Experiment 4, the Irish dark peat/perlite mix also supported the establishment of AMF root colonisation of the strawberry plugs. However, differences among the five AMF species inoculated were evident (Table 3.5; $F_{4,78}$ = 19.6; P < 0.001) and average % RLC was in the order: *R. irregularis* (64 %) > *F. mosseae* (42 %) and *G. microagregatum* (40 %) > *C. claroideum* (26 %) > *F. geosporum* (12 %). A significant effect (Table 3.5; $F_{3,78}$ = 3.5; P = 0.018) of strawberry cultivar on % RLC was also detected as RLC for the strawberry cultivars 'Elsanta' 'Red Glory' and 'Vibrant' was ca. 40%, but Malling Centenary' only 27 %. There was no significant difference of % AMF between the two plant runner size groups (Table 3.5) and there was no significant interaction among factors (Table 3.5).

Table 3.5. Three-way ANOVA of the total percentage root colonisation by arbuscular mycorrhiza fungi (% RLC) in strawberry plugs grown for 7 weeks in Irish dark peat/perlite mix (7:3, v/v) under glasshouse conditions (Experiment 4). Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	**% RLC**			
	df	**Mean square**	***F***	***P***
Cultivar	3	0.23	3.54	**0.018**
AMF	4	1.28	19.62	**< 0.001**
Plant (runner) size	1	0.06	0.92	0.340
Cultivar × AMF	12	0.08	1.21	0.294
Cultivar × Plant (runner) size	3	0.10	1.58	0.201
AMF × Plant (runner) size	4	0.01	0.09	0.986
Cultivar × AMF × Plant (runner) size	12	0.03	0.51	0.902
Residual	78	0.07		

df = degree of freedom. Fixed effects include cultivar (refers to 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'), AMF (refers to plants inoculated singly with the AMF species: *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Funneliformis geosporum*, *Glomus microagregatum*).

In Experiment 5, AMF colonisation of roots of strawberry plugs cultivated in peat/perlite mix grown under glasshouse/poly-tunnel conditions was observed. DSE were also present in the roots. Before cold storage % RLC and % DSE colonisation ranged from 1-16 %, and 11-16 % respectively (Table 3.6).

Table 3.6. Percentage of root length colonisation by arbuscular mycorrhiza fungi (AMF) and by dark septate endophytes (DSE) of *Fragaria* x *ananassa* plug cv. 'Vibrant' and 'Red Glory' inoculated with single AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis*, or *Claroideoglomus claroideum*) after 113 d of cultivation in the Irish dark peat/perlite mix (7:3, v/v) under glasshouse/poly-tunnel conditions.

Plant cultivar	**AMF species**	**% RLC***	**% DSE***
Vibrant	*F. mosseae*	1 ± 1	12.3 ± 1.9
	R. irregularis	15.7 ± 2.3	14.3 ± 2.3
	C. claroideum	8.7 ± 2.6	12.3 ± 0.9
Red Glory	*F. mosseae*	15.3 ± 4.7	15.7 ± 3.8
	R. irregularis	4.3 ± 1.8	14.7 ± 1.9
	C. claroideum	9.7 ± 4.5	11.3 ± 3.5

* mean ± SE; *n* = 3, each root sample was pooled from five individual plants

3.3.2 Effect of AMF on strawberry plug transplant growth

In Experiment 1, all plants survived and grew normally. Plant crown diameter was affected by AMF inoculation ($F_{1,56}$ = 11.7; P = 0.001). AMF inoculated plants had bigger crowns (mean = 3.2 ± 0.1 mm, n = 36) than the non-AMF treated plants (mean 2.7 ± 0.1 mm; n = 24). But for plant height, there were not significant AMF effects. Plant fresh biomass was influenced by AMF inoculation ($F_{1,56}$ = 5.8; P = 0.021). AMF-inoculated plants produced less fresh biomass (mean 2.4 ± 0.1 g; n = 36) than the non-mycorrhizal treatment (mean 2.7 ± 0.1 g; n = 24).

In Experiment 2, despite the infestation by spider mites, no plants died or showed visual differences in terms of growth. Plant crown diameter was not affected by AMF inoculation or by the weaning substrate. The interaction between the two factors was not significant (Figure 3.16A; $F_{1,78}$ = 3.89; P = 0.052). Plant fresh biomass was not affected by AMF inoculation or by the weaning substrate and neither was the interaction term (Figure 3.16A). Plant dry biomass did not vary between AMF and non-mycorrhizal plants. Similarly the weaning substrate did not affect the plant dry biomass. However, a significant interaction between AMF and substrate was detected for plant dry biomass (Figure 3.10C; $F_{1,78}$ = 3.9; P = 0.033). Finally, shoot/root weight ratio was significantly affected by the substrate (Figure 3.10D; $F_{1,78}$ = 16.1; P < 0.001). This ratio was smaller in coir (mean = 4.4; SE ± 0.1; n = 42) than in vermiculite (mean = 5.1; SE ± 0.1; n = 42).

In Experiment 3, all plants appeared to be healthy. Plant crown diameter was not affected by AMF inoculation but by plant cultivar (Figure 3.11A; $F_{1,89}$ = 9.5; P = 0.003). Average crown size of ‘Vibrant’ (mean = 6.8 mm; SE ± 0.1; n = 47) was bigger than ‘Red Glory’ (mean = 6.4 mm; SE ± 0.1; n = 50). The interaction between AMF treatment and cultivar was not significant. Similarly, plant height was not affected by AMF inoculation but by plant cultivar (Figure 3.11B; $F_{1,89}$ = 8.5; P = 0.004) with ‘Vibrant’ plants taller (mean = 89.2 mm; SE ± 2.2; n = 47) than ‘Red Glory’ (mean = 81.1 mm; SE ± 1.8; n = 50). There was no interaction between factors AMF and cultivar.

In Experiment 4, both plant cultivars (GLM; P = 0.023) and plant runner-tip size (GLM; P < 0.001) had significantly affected plant survival (Table 3.7). The dead plants came mostly from the plant size ‘Small’ and cultivar ‘Malling Centenary’. Mycorrhiza inoculation did not affect plant crown size or plant survival (Table 3.7). In addition, they were no interactions between the three factors (Table 3.7).

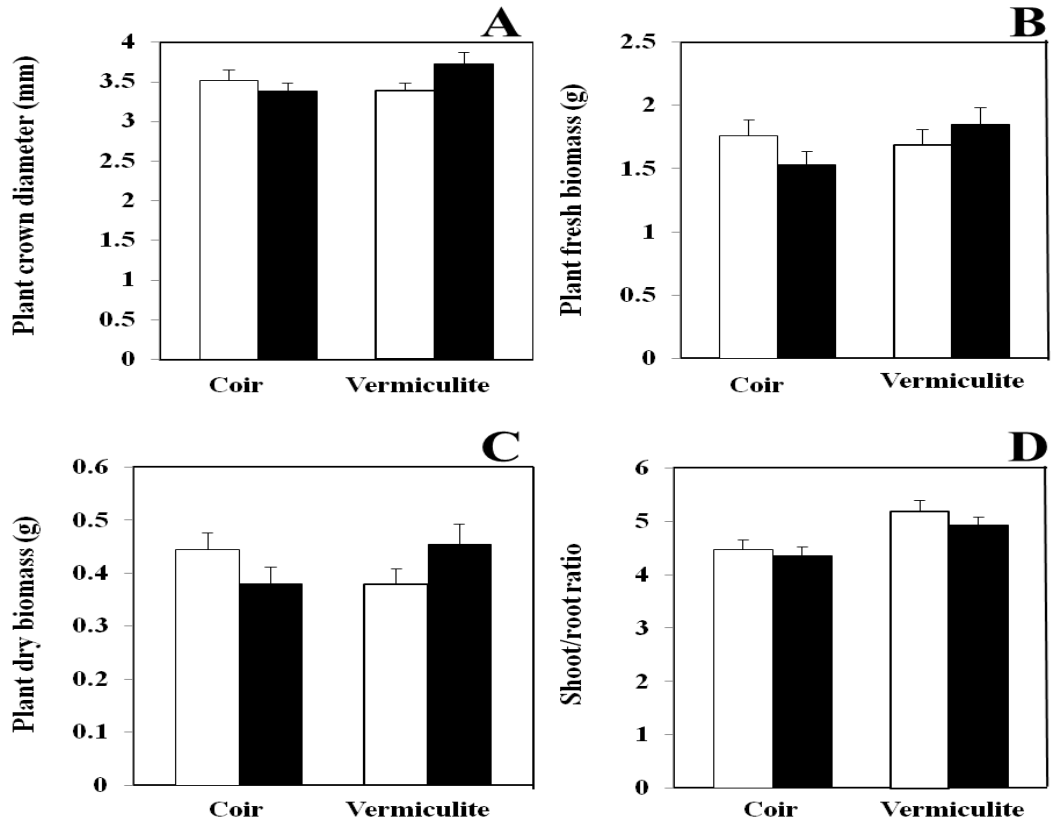

Figure 3.10. (A) Mean plant crown diameter (mm), (B) mean plant fresh biomass (g), (C) mean plant dry biomass (g) and (D) Shoot/root weight ratio in Experiment 2. NM, non-mycorrhizal (white bars) and AMF inoculated (black bars) treatments. Bars represent standard error (SE). For all treatments $n = 21$. (C) For plant dry biomass, the interaction between AMF and substrate ($F_{1,78} = 3.9$; $P = 0.033$) was significant. (D) Shoot/root wright ratio was significantly smaller in coir compare to vermiculite ($F_{1,78} = 16.1$; $P < 0.001$).

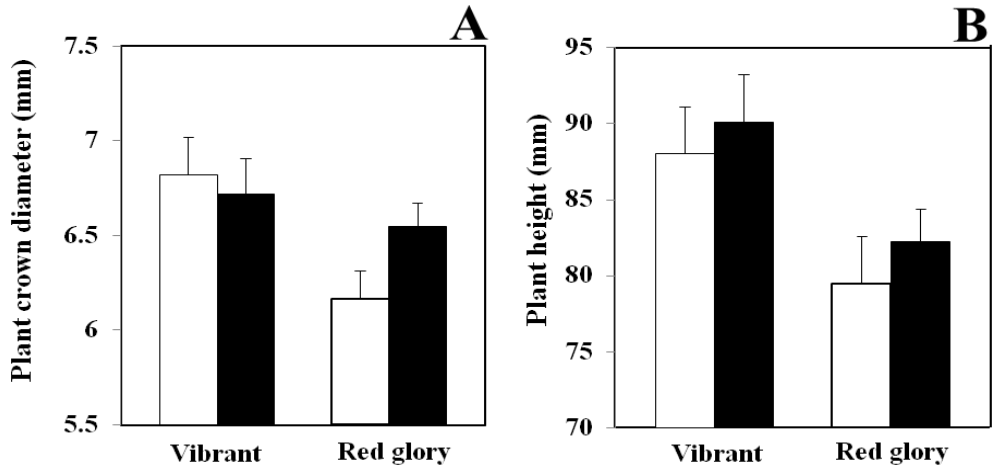

Figure 3.11. (A) Mean plant crown diameter (mm) and (B) mean plant height (mm) in Experiment 3. Non-mycorrhizal and AMF inoculated treatments are represented by the white and black bars, respectively. Bars represent mean + 1 SE. For non-mycorrhizal treatment n = 20 and for AMF inoculated treatment n = 27-30. Cultivar had significant effects on (A) crown diameter ($F_{1,89} = 9.5$; $P = 0.003$) and (B) height ($F_{1,89} = 8.5$; $P = 0.004$).

Table 3.7. Results of three-way ANOVA of plant crown diameter (mm) and generalised linear model (GLM) of survival (%) of plants pre-inoculated with AMF and grown for 7 weeks in the Irish dark peat/ perlite mix (7:3, v/v) under glasshouse conditions (Experiment 4). Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	**Plant crown diameter (mm)**				**Plant survival (%)**		
	df	**Mean square**	***F***	***P***	**df**	***deviance***	***P***
Cultivar	3	49.5	24.2	**< 0.001**	3	9.5	**0.023**
AMF	1	2.7	1.3	0.251	1	2.1	0.146
Plant (runner) size	1	215.4	105.4	**< 0.001**	1	26.2	**< 0.001**
Cultivar × AMF	3	1.9	0.9	0.427	3	6.3	0.097
Cultivar × Plant (runner) size	3	1.4	0.7	0.555	3	6.2	0.103
AMF × Plant (runner) size	1	0.6	0.3	0.578	1	9.7	0.458
Cultivar × AMF × Plant (runner) size	3	0.5	0.2	0.872	3	0.9	0.446
Residual	239	2.0			271	125.4	

df = degree of freedom. Fixed effects include cultivar (refers to ‘Elsanta’, ‘Malling Centenary’, ‘Red Glory’ and ‘Vibrant’), AMF (refers to plants inoculated with AMF) and runner-tips size (‘Large’ and ‘Small’).

3.3.3 Effect of cold storage on AMF and DSE survival

After a month of growth under growth room conditions, microscopic assessments of newly formed strawberry roots showed the presence of fungal structures for both cultivars ('Vibrant' and 'Red Glory') irrespective of the length of cold storage (up to 5 months; Figure 3.12; 3.13). The presence of arbuscules and/or vesicles confirmed the presence of AMF (Figure 3.12). The presence of microsclerotium-like structures and septate hyphae in the root cortex indicated the presence of DSE (Figure 3.13A-F). In addition, smaller microsclerotia and moniliform group of fungal cells coexisting with dark septate hyphae were occasionally observed (Figure 3.13B, E, F), suggesting that different species or developmental stages of DSE were present inside the strawberry roots. Sometimes, there was overlapping of AMF and DSE structures, but this occurred rarely.

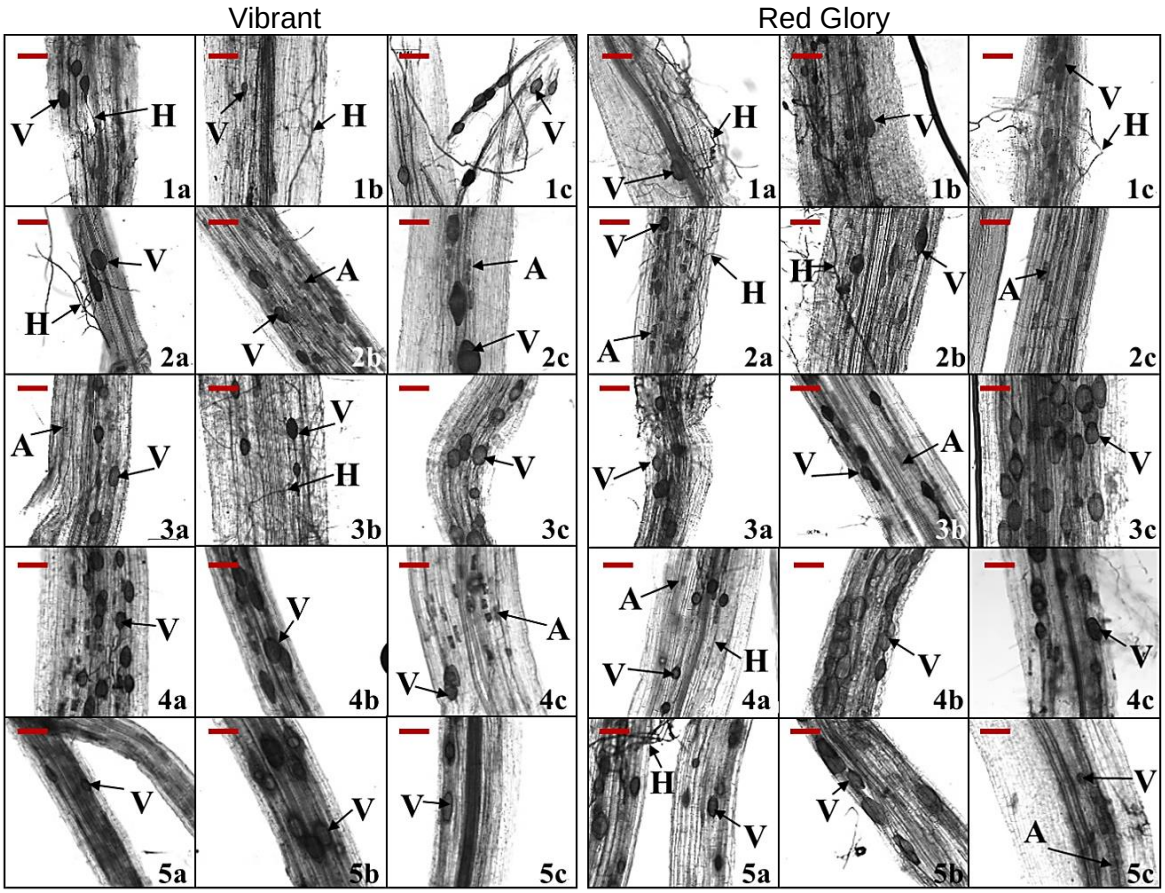

Figure 3.12. Root colonisation by arbuscular mycorrhiza fungi (AMF) of *Fragaria* x *ananassa* plug cv. 'Vibrant' (left picture block) and 'Red Glory' (right picture block) after 30 d of cultivation in autoclaved attapulgite clay in a growth chamber at 22 °C. Numbers represent the duration (months) in which plants were cold stored at -2 °C: (1) one month, (2) two months, (3) three months, (4) four months and (5) five months. Longitudinal squash of roots stained with trypan blue colonisation by single AMF species: (a) *Funneliformis mosseae*; (b) *Rhizophagus irregularis*; (c) *Claroideoglomus claroideum*. Letters next to black arrows are A: arbuscule, V: vesicle, H: hypha (red scale bars represent 100 µm).

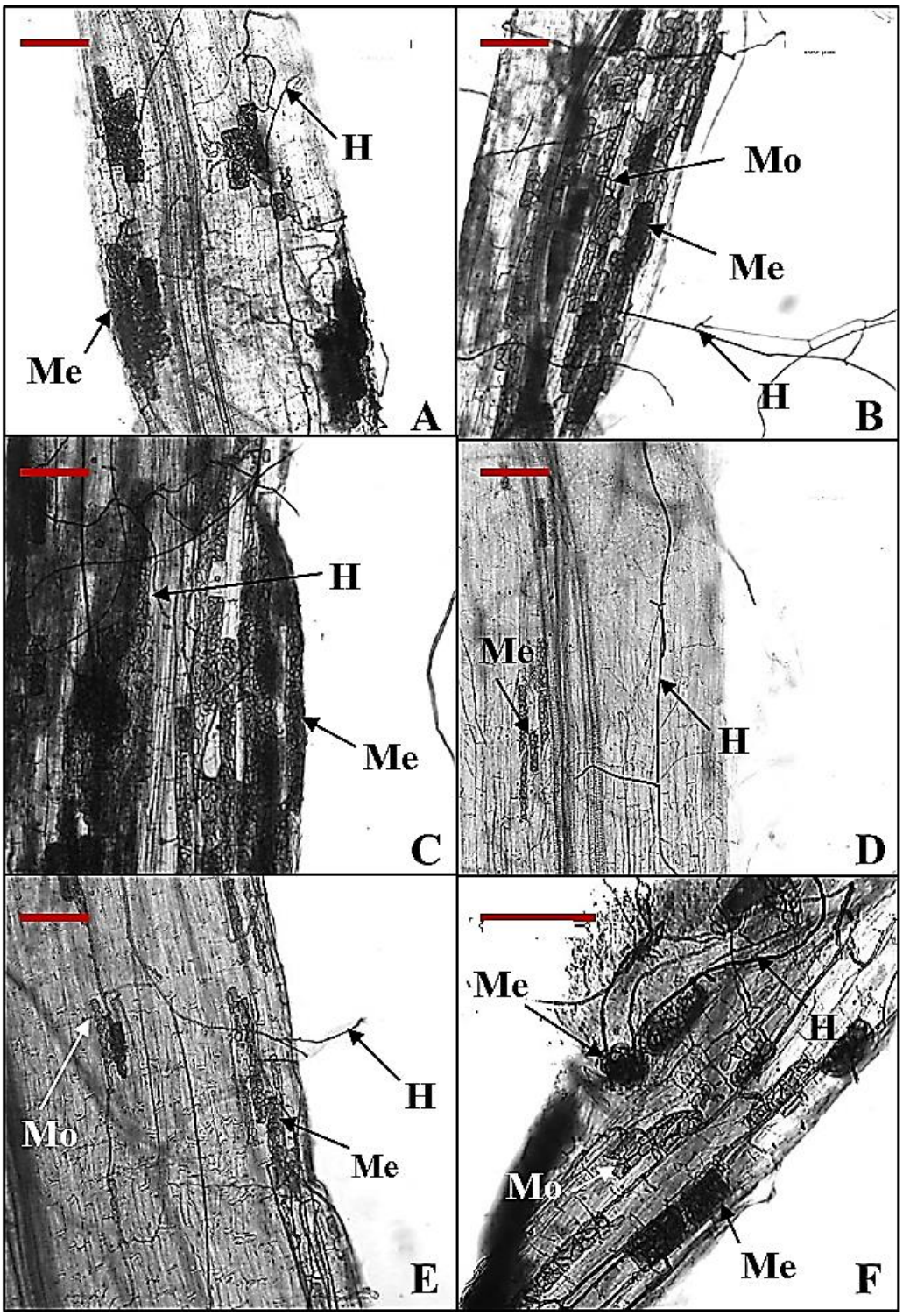

Figure 3.13. Root colonisation by dark septate endophytes (DSE) of *Fragaria* x *ananassa* plug cv. 'Vibrant' (A-C) and 'Red Glory' (D-F) cold stored at -2 °C for 2 months, after 30 d of cultivation in autoclaved attapulgite clay in a growth cabinet at 22 °C. Similar structures were observed in roots of plants cold stored at -2 °C for 1, 3, 4 and 5 months (data not shown). Letters next to black arrows are H: hypha, Mo: moniliform cells and Me: microsclerotium (red scale bars represent 100 µm).

3.3.4 Effect of DSE and cold storage on AMF root colonisation level

There was a negative correlation between % RLC and % DSE root colonisation ($F_{1,88}$ = 127.1, P < 0.001, r = -0.59; Figure 3.14). Strawberry plugs that were stored for five months at -2 °C had the highest AMF colonisation level (mean = 57.6, ± 4.0%, n = 18 ; Figure 3.15), whereas the plugs stored for one month had the lowest (mean = 24.1 ± 4.3%,, n = 18; Figure 3.15). DSE colonisation was lowest in the strawberry plugs that were stored for five months at -2 °C (mean = 15.4 %, SE ± 0.025, n = 18) and highest in plants stored for one month (mean = 51.2 %, SE ± 0.039, n = 18) (Figure 3.15). When DSE was included as a covariate in the three-way ANOVA, there were significant effects of cold-storage duration ($F_{4,59}$ = 2.8, P = 0.032), AMF species ($F_{2,59}$ = 8.3, P < 0.001) and the 3-way interaction between duration × cultivar × AMF interaction ($F_{8,59}$ = 2.5, P = 0.023) on AMF colonisation (Table 3.8). AMF colonisation differed significantly only between the plants cold stored for one and four months at -2 °C. Overall, plants pre-inoculated with *C. claroideum* (mean = 53.7 %, SE ± 2.9, n = 30) had a significantly higher level of colonisation than the plants pre-inoculated with *F. mosseae* (mean = 34 %, SE ± 4.0, n = 30) or *R. irregularis* (mean = 34.5 %, SE ± 0.034, n = 30) regardless the cultivars and duration of cold storage.

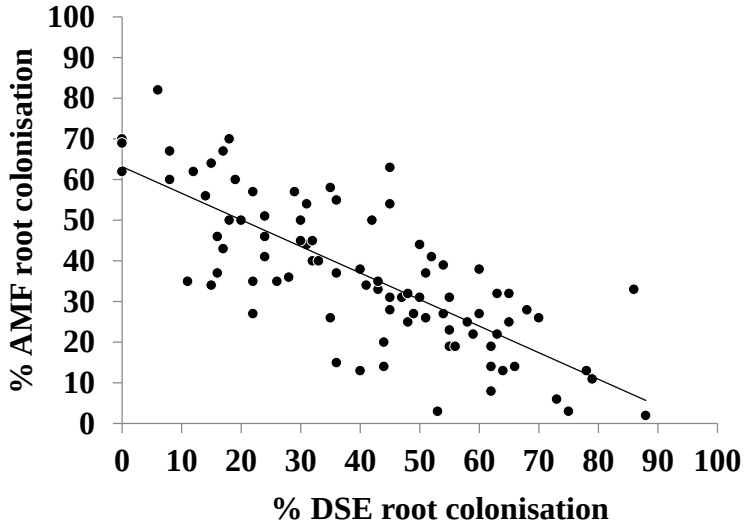

Figure 3.14. Relationship between the percentage arbuscular mycorrhiza fungi root colonisation (% AMF) and percentage dark septate endophytes root colonisation (% DSE): r = -0.59, P < 0.001.

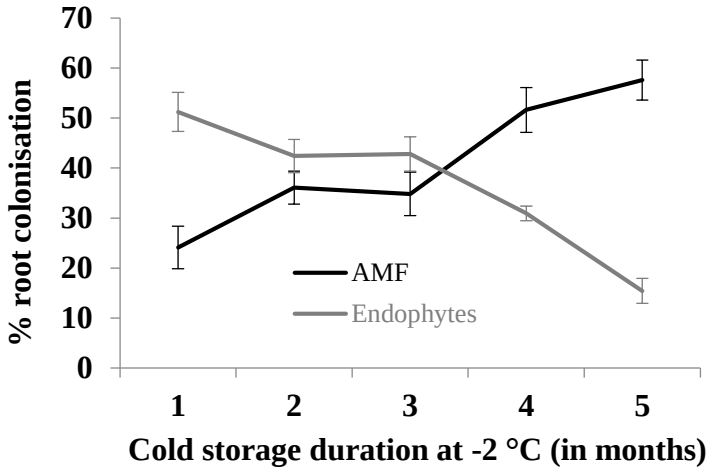

Figure 3.21. Percentage arbuscular mycorrhiza fungi (% AMF) and dark septate endophytes (% DSE) root length colonisation means (± SE, *n* = 18; each root sample was pooled from three individual plants) after different cold storage duration (one to five months) followed up by 30 d of re-growth in a growth room at 22 °C.

Table 3.8. Results of the three-way ANOVA of % arbuscular mycorrhiza fungi (% RLC) colonisation levels in strawberry roots pre-inoculated with AMF, cold stored at -2 °C for one to five months and re-grown for 30 d in a growth room at 22 °C. Significant differences are shown by bold font ($P \leq 0.05$).

Source of variation	**df**[a]	**Mean square**	***F***	***P***
Duration (of cold storage)	4	0.051	2.8	**0.032**
Cultivar	1	0.003	0.2	0.699
AMF	2	0.149	8.4	**<0.001**
Duration × Cultivar	4	0.023	1.3	0.293
Duration × AMF	8	0.034	1.9	0.079
Cultivar × AMF	2	0.019	1.1	0.356
Duration × Cultivar × AMF	8	0.043	2.5	**0.023**
Covariate (DSE colonisation)	1	0.695	39.2	**<0.001**
Residual	59	0.018		

[a]Df = degree of freedom. Five cold-storage durations (1, 2, 3, 4 and 5 months) at -2 °C), cultivar ('Vibrant' and 'Red Glory'), and AMF species inoculated (*Funneliformis mosseae* or *Rhizophagus irregularis* or *Claroideoglomus claroideum*); DSE colonisation percentage was included as a covariate.

3.4 Discussion

3.4.1 Influence of weaning conditions on AMF colonisation

The soil-less substrates used for weaning, propagation and/or cultivation of horticultural crops, including strawberry, usually lack AMF (Azcón-Aguilar and Barea 1997, Vestberg et al. 2004a). By introducing AMF at a very early stage of the strawberry propagation process, it might be possible to decrease fertiliser and pesticide application rates without adverse effects on plant growth and health. This study represents the first attempt to examine the combined effects of high moisture conditions and soil-less substrates on strawberry root colonisation by AMF during the weaning phase. The results indicated that AMF can colonise roots of strawberry plugs under damp condition in soil-less substrates.

Plant propagators or misting systems are required to maintain damp condition for at least two weeks to ensure strawberry plant acclimatisation and rooting (Durner et al. 2002, Treder et al. 2015). Previous evidence suggests AMF root colonisation is limited under damp condition and declines with an increase in the amount of water in the substrate due to lower oxygen availability (Khan and Belik 1995, Muthukumar et al. 1997, Thormann et al. 1999, Miller 2000). Even though the soil-substrates tested (i.e. coir, peat/ perlite mix and vermiculite) were wet during the weaning period, AMF could successfully colonise the strawberry plug roots. These results agree with the literature reporting AMF presence in wet land habitats (Søndergaard and Laegaard 1977, Miller 2000) or successful root colonisation of crops under irrigation (Baslam et al. 2011). Hence, AMF symbiosis establishment is possible during the weaning stage of microplants and runner derived strawberry plants.

Microplants inoculated with AMF and grown in vermiculite (Experiment 1 and 2) showed the highest level of AMF colonisation (80-90 %). Thus, use of vermiculite as a soil-less substrate is conducive for establishment of the AMF symbiosis in the roots of strawberry microplants. This agrees with previous reports describing vermiculite as a suitable substrate for commercial AMF inocula production (de Santana et al. 2014, Rouphael et al. 2015). Several other studies have successfully colonised strawberry plants with AMF using vermiculite as amendment in their potting mixes (Mark and Cassells 1996, Murphy et al. 2000a, Sinclair et al. 2013).

In Experiment 2, strawberry plantlets were successfully colonised by AMF in coir, which agrees with the findings of other studies (Linderman and Davis 2003a, Robinson Boyer et al. 2016). Interestingly, the level of colonisation obtained in vermiculite was twofold higher (80 %) than in coir (40 %). Robinson Boyer et *al*. (Robinson Boyer et al. 2016) reported that for both maize and strawberry plants AMF colonisation coir is less than in attapulgite clay (a soil-less substrate also known to be very conducive for AMF (Vestberg and Kukkonen 2007, Leigh et al. 2009)), although colonisation of maize by AMF in coir is still much greater than in strawberry. Therefore, lower levels of AMF colonisation in coir could indicate that (1) coir is not conducive

for AMF to colonise roots, (2) movement of inoculum in coir is limited, and (3) spore production is reduced. Further studies are required to understand the interaction between strawberry, AMF and the properties of coir.

Peat based mixes are commonly used by nurseries as a substrate to propagate strawberry plants (Vestberg et al. 2000, D'Anna et al. 2002, Treder et al. 2015). The results of this study demonstrated that different AMF species could colonise strawberry roots in an Irish peat/perlite mix (7:3 v/v) and are in contrast to the frequently reported negative effects of peat-based substrates on AMF propagules and reduced root colonisation for strawberry and other horticultural plants commonly reported in the literature (Vestberg et al. 2000, Linderman and Davis 2003a, b, Vestberg et al. 2004a, Vestberg and Kukkonen 2007). Surprisingly, the % RLC in Experiment 5 (1-16 %) was lower compare to Experiment 3 and 4 (40-81 %). Several factors may explain this result. Firstly, the AMF inoculants used had a much lower number of infective propagules (23-170 propagule mL^{-1} cf. 1600 propagule mL^{-1}). By increasing the number of infective propagules added to the strawberry plugs, similar colonisation percentage might have been obtained, assuming that the 'colonisation capacity' (Tommerup 1992) is similar between *F. mosseae*, *R. irregularis* and *C. claroideum*. Secondly, the lower AMF colonisation level may be related to the growing season. In Experiment 5, the runner-tips were inoculated with AMF at the end of September, while in the other trials the plants were inoculated between March-June. The growing conditions were also relatively different in Experiment 5. The plants had an extended growing period (18 weeks cf. 6-8 weeks in Experiment 3 and 4). The plugs were grown under glasshouse conditions and then under poly-tunnel conditions (with natural light and cooler temperatures). In addition, plant roots were sampled in January (during vegetative dormancy), while plant roots were sampled between April-July (months of maximum vegetative growth) in the other experiments. Seasonal variations of AMF colonisation were previously documented under glasshouse (Niemi and Vestberg 1992) and field conditions (Branzanti et al. 2002). Nevertheless, additional studies are needed in order to characterise the effects of the physical, chemical and biological properties of peat-based substrates and investigate which are the exact factors responsible for the variations in root colonisations.

Microscopic assessments showed that strawberry plug roots were sometimes colonised by DSE (Experiment 4 and 5). The DSE inocula could originate from the Irish dark peat used during plant weaning stage. This is supported by the fact that DSE were not observed in the other experiments in which other soil-less substrate was used. Occurrence of DSE has mostly been described from soil systems (Wagg et al. 2008, Lizarraga et al. 2015, Vandegrift et al. 2015) but has also been reported from peat (Fuchs and Haselwandter 2004, Thormann 2006, Weishampel and Bedford 2006). The interest in DSE has recently increased but thus far only 30 DSE species have been identified and their phylogenetic identity or functional roles are

uncertain (Andrade-Linares and Franken 2013, Knapp et al. 2015). In the present investigation, the DSE observed in the strawberry roots were unidentified. Future studies on phylogenetic analysis of DSE in strawberry root and their potential effect on plant growth may provide insight into their function and identity.

3.4.2 Effect of strawberry cultivar and plant size on root colonisation level

Different strawberry cultivars were tested in combination with various AMF species to study the potential presence of specific interactions between the host and the fungal symbionts (Experiment 3 and 4). Results showed that AMF species generally do not differ in their ability to colonise strawberry cultivars.

In Experiment 3, there was no cultivar effect on AMF colonisation. This contrasts with the results from Experiment 4 where the 'Malling Centenary' cultivar had lower colonisation levels overall, while 'Elsanta', 'Red Glory' and 'Vibrant' had similar levels of AMF colonisation. Variability of AMF colonisation among strawberry cultivars grown under field conditions has previously been reported (Robertson et al. 1988). Moreover, studies conducted under glasshouse conditions showed that strawberry cultivars were either colonised equally (Robertson et al. 1988) or differentially (Chávez and Ferrera-Cerrato 1990, Vestberg 1992c) by AMF. The reasons for the cultivar effect in Experiment 4 are unknown. Previous reports have also highlighted the presence of a relationship between root morphology and mycotrophy with AMF (Tawaraya 2003). The root architecture of different strawberry cultivars and their root exudates composition have been reported to vary greatly (Vestberg 1992c). The lack of AMF colonisation of some wheat varieties has previously been linked to the absence of root sugar exudates (Azcon and Ocampo 1981). Therefore, a potential difference in root morphology and/or qualitative/ quantitative differences in root exudates of 'Malling Centenary' may have influenced rhizosphere microbial communities or signalling pathways affecting AMF colonisation. The tripartite interaction between host plant, AMF and other microbes have been reported to influence positively (Rouphael et al. 2015) or negatively AMF root colonisation (Germida and Walley 1996). Characterisation of soil microbial community function remains at present limited, but the enhanced application of metagenomic tools will allow us to access such information in a near future.

The results from Experiment 4 suggested that AMF species do not differ in their capacity to colonise roots of different sized plants; both plant size groups were colonised equally by AMF. This finding agrees with a previous report showing that total root colonisation by AMF and DSE were not affected by the difference in size of *Gentian Ella campestris* (L.) Börner plants (Piippo et al. 2011).

3.4.3 Effect of AMF pre-inoculation on strawberry plug growth

We showed that strawberry microplants and runner-tip derived plants could be AMF colonised during the weaning stage, however the results indicated that early AMF inoculation does not necessarily increase plugs growth during propagation and indeed could even lead to growth reductions. AMF colonisation reduced the total fresh biomass of microplants (Experiment 1), perhaps due to conditions non-optimal conditions for photosynthesis resulting in a carbon drain on the host plant (Bethlenfalvay et al. 1982). Despite the reduction of fresh weight, plants crown size was significantly increased by AMF inoculants although the reason for this is currently unclear. AMF may have resulted in changes in hormonal and nutritional conditions but this would require further investigation. In a similar experiment (Experiment 2), AMF colonisation did not affect plant crown size or total fresh biomass, but a significant interaction between AMF and substrates on the total plant dry biomass was observed. In coir, AMF root colonisation with *R. irregularis* reduced total plant biomass, whereas in vermiculite the AMF increased the total plant biomass.

In peat-based substrate AMF had neutral effect on runner-derived plants. In contrast, another study highlighted that certain combinations of strawberry cultivars and AMF species resulted in the increase of plant biomass in peat based substrate (Vestberg 1992c). Perhaps, the neutral effect of AMF inoculation observed in the current experiment could be explained by the fact that nutrients were not limiting in the Irish dark peat/perlite mix used and/or plant growth window may have been also too short to detect an effect.

3.4.4 Effect of cold storage on the presence of AMF after plug re-growth

Experiment 5 was the first study to explore the effect of cold storage on the survival of AMF in pre-inoculated strawberry plug roots. The results indicated that propagules of *F. mosseae*, *R. irregularis*, *C. claroideum* were able to survive and to retain their infectivity after several months of cold storage. This result is in agreement with previous studies in which *Glomus* species propagules remained infective in frozen soil over winter (Addy *et al*., 1994; Addy *et al*., 1997) or after artificial freezing treatment at -12 °C for 7 d in soil, 48 h in M medium Addy *et al*., 1998) and at -5 °C for 4 weeks in silica sand (Klironomos *et al*., 2001). Addy *et al*. (1997) reported that extraradical mycelium of *R. irregularis* and *G. fasciculatum* survived freezing treatment and mycelia were much effective as inoculum compared to the spores of the same fungi. Therefore, AMF propagules can survive freezing temperature for several months given the environment in which they inhabit.

3.4.5 Effect of DSE and cold storage on AMF root colonisation level

Microscopic assessments showed that strawberry plug roots grown in peat were regularly associated with DSE. These DSE fungi have been described as 'miscellaneous fungi' that colonise the root tissue of a large array of plant species (Jumpponen and Trappe 1998) without causing any noticeable damage to their host (Jumpponen 2001) and have been frequently reported to co-exist with AMF (Urcelay 2002). Lizarraga *et al.* (2015) reported the co-existence of DSE and AMF in *F.* x *ananassa* grown under field conditions. Hence, it is reasonable to expect that as AMF and DSE share the same spatial niche they may interact with each other. The present study showed a negative relationship between % AMF and % DSE root colonisation suggesting niche competition for space. Microscopic analysis revealed that strawberry roots heavily colonised by DSE rarely contained mycorrhizal structures. This observation agrees with previous reports showing that plants with the highest AMF colonisation generally showed the lowest DSE colonisation and *vice versa* (Kandalepas et al. 2010, Urcelay et al. 2011). Additional studies are need to characterise the nature of AMF and DSE interaction in strawberry roots (e.g. competition for spaces, for carbon and/or mineral nutrients, and effect of DSE exudates) as well as their effects on the host fitness.

Root colonisation by AMF was shown to increase after prolonged storage at -2 °C (e.g. 24.1 % after 1 month c.f. 57.6 % after 5 months and negatively correlated with DSE colonisation. Therefore, DSE root colonisation level was included as a covariate in ANOVA to remove its influence on AMF root colonisation. Against all expectations the colonisation by AMF remained different between plants cold stored for 1 and 4 months. Hence, the decline of DSE was not the only factor responsible for the increase of AMF root colonisation over time. Perhaps, prolonged cold storage resulted in the breakage of spore dormancy that might have resulted in additional spore germination and root colonisation. This hypothesis is supported by a previous work showing that *F. mosseae* and *G. fasciculatum* spores cold stored for 28 d at -10 °C (Safir et al. 1990) were reported to break AMF spore dormancy and synchronise germination. Hence, additional experiments should be conducted in absence of DSE to verify if a significant increase of AMF root colonisation level occurs again and characterise the nature of this significant increase of root colonisation.

Additionally, AMF species inoculated responded differently to freezing temperature. The plants pre-inoculated with *C. claroideum* (54 %) presented a significantly higher level of root colonisation compared to *R. irregularis* (35 %) and *F. mosseae* (34 %). Therefore, assuming that the three AMF species tested had similar colonisation capacities, it was hypothesised that cold storage resulted in a better breakage of *C. claroideum* spore dormancy or tolerance to cold damages compared to spore of *R. irregularis* and *F. mosseae*. Previous studies have

highlighted that spore dormancy varies greatly between AMF species, even under laboratory conditions (Juge et al. 2002).

4 The potential of pre-colonised strawberry plugs with mycorrhizal inoculants to increase tolerance to *Verticillium* wilt

The aim was to investigate whether strawberry plug plants pre-inoculated with commercially available AMF could enhance plant growth and reduce strawberry wilt incidence in contaminated soils. Several strawberry cultivars susceptible to *Verticillium* wilt were screened in this study to test the hypothesis that pre-inoculation with AMF can promote plant growth and increase tolerance to strawberry wilt after transplantation under glasshouse or open field conditions.

4.1 Materials and methods

A total of six experiments were conducted in 2014-2015. Two experiments were carried out with wilt conidial suspension and one experiment with mycelia under controlled conditions. However, none of these inoculations resulted in wilt development; hence are not discussed further. The other three experiments were carried out with contaminated soils under glasshouse (Figure 4.1A) or open field (Figure 4.1B) conditions as summarised in Table 4.1.

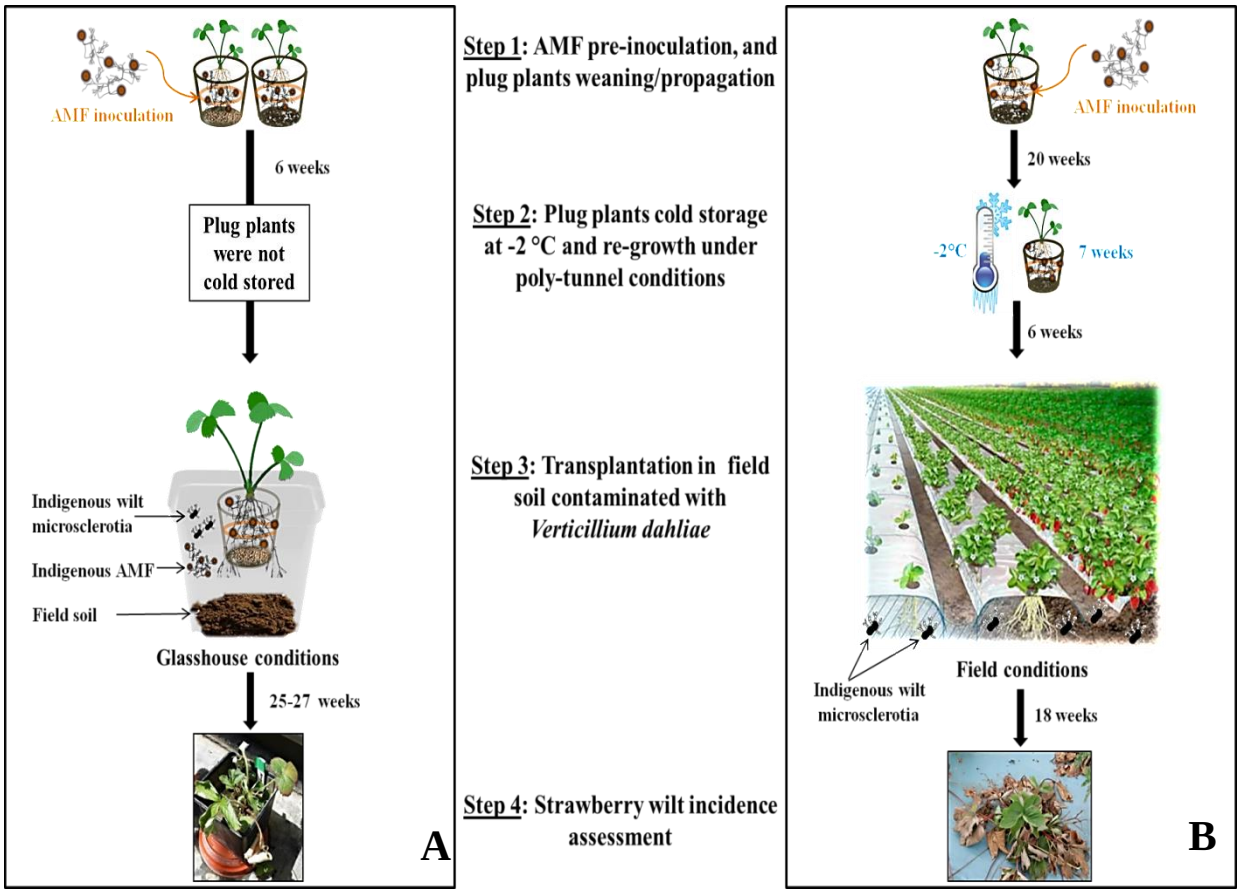

Figure 4.1. Schematic representation of the experimental setup to study the effect of arbuscular mycorrhiza fungi (AMF) pre-inoculation against Verticillium dahliae: (A) glasshouse

experiments (Experiment 1 and 2) and (B) field experiment (Experiment 3). Microplants or runner-tips were AMF inoculated and propagated in soil less-substrates (Step 1). Plugs were cold stored for seven weeks at -2 °C only for Experiment 3, while plugs were kept under glasshouse conditions for Experiment 1 and 2 (Step 2). Plugs were transplanted in field soil contaminated with V. dahliae microsclerotia (Step 3) and disease development assessed (Step 4).

Table 4.1. Summary of the three experiments to study the effect of early arbuscular mycorrhizal fungi (AMF) colonisation of strawberry plug transplants to control Verticillium dahliae.

AMF treatment	**Experiment number**		
	1	**2**	**3**
Total no. treatments	5	10	12
AMF species and controls[a]	*F.m*, *R.i*, *C.c,* Cb^-, Cb^+	*F.m*, *R.i*, *C.c*, Cb^-, Cb^+	*F.m*, *R.i*, *C.c*, Cb^+
Plant cultivars[b]	EM-1996	RG, V	MC, RG, V
Total no. of replicates	12	10	96
No. blocks	3	5	6
Type of plant material	Plug plant	Plug plant	Cold stored plug plant
Location	Glasshouse	Glasshouse	Field
Pathogen propagule density[c]	22.2 CFU g^{-1} of soil	22.2 CFU g^{-1} of soil	1.9 CFU g^{-1} of soil
Start date (i.e. transplantation)	19/03/14	16/04/14	22/05/15
Experimental duration	27 weeks	25 weeks	18 weeks

[a] Abbreviations *F.m*, *R.i*, *C.c*, *G.m* and *F.g* respectively stand for the single AMF species inoculated: *Funneliformis mosseae*, *Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*, *Funneliformis geosporum*. Cb^-: non-AMF control inoculated with autoclaved attapulgite clay; Cb^+: non-AMF control inoculated with an autoclaved equal mix of AMF species used and inoculated with bacterial washing

[b] Abbreviations E, MC, RG, and V respectively stand for strawberry cultivars: 'Elsanta', 'Malling Centenary', 'Red Glory' and 'Vibrant'

[c] The pathogen propagules were microsclerotia naturally present in the field soil. CFU stands for colony-forming unit

4.1.1 Plant materials

All strawberry cultivars used are known to be susceptible to V. dahliae. The AMF pre-colonised strawberry plug transplants used in Experiment 1 and 2 derived from plugs produced in Chapter 3 (see Section 3.2.2). For Experiment 3, AMF pre-colonised strawberry plugs were produced as described in Section 3.2.2 with the following modifications: (1) after cultivation for 82 d under glasshouse conditions, the plants were transferred to a poly-tunnel with natural shorter days and cooler temperatures for 76 d to induce dormancy, (2) plants were then acclimated to lower temperatures for 8 d in the dark at 2 °C and (3) plants were then cold

stored at -2 °C for 7 weeks. At the end of the cold storage period, these plants were transferred to a poly-tunnel for six weeks in the spring to induce plant growth before transplantation to the open field.

4.1.2 Determination of wilt inoculum density in field soils

Estimation of *V. dahliae* microsclerotia concentration in the soils was carried out using the method of (Harris et al. 1993). In Experiment 3, soil samples deriving from each of the 72 plots of the experiment was pooled together for this analysis. Hereafter, the microsclerotia concentrations are expressed in colony-forming unit (CFU) g^{-1} of soil.

4.1.3 Plug transplantation in wilt contaminated soils

A randomised block design was used in all experiments (Table 4.1). For Experiment 1 and 2 plugs were re-potted into 1 L plastic pots (11 x 11 x 12 cm, Desch Plantpak Ltd, Essex, UK) filled up with soil collected in a non-fumigated commercial strawberry field at EMR, UK (N 51°17'20.93'', E 00°27'11.52''; soil: Barming series, loamy fine sand). The background nutrient status of the field soil was analysed before plant transplantation (Table 4.2). The soil was sieved beforehand with a garden riddle (square hole wire mesh ca. 5 mm). Plug plants were then kept under standard greenhouse conditions (22-23 °C, ca. 40 % RH, natural light:dark cycle, adequate pest control; Figure 4.2A, B) as outlined in Table 4.1. Plants from Experiment 1 and 2 were watered daily with tap water and fertilised once a week with 50 mL of full-strength Rorison's nutrient solution but with phosphate omitted (Hewitt and Bureaux 1966). In order to induce a moderate water stress to encourage wilt development for the last seven week period of Experiment 1 and 2, plants were watered only two or three times a week (one of those watering event included fertilisation).

Experiment 3 was carried out in raised double-row beds at EMR, UK (N 51°17'19.90'', E 00°27'13.38'' soil: Barming series, loamy fine sand). The background nutrient status of the field soil was also analysed before transplantation (Table 4.2). The soil was not fumigated before planting. Plastic drip irrigation was laid down in the middle of the bed, which was covered with blue plastic mulch (Figure 4.2C, D). A spacing of 30 cm between rows and between plants was used. There was a spacing of ca. 100 cm between neighbouring plots (each containing 16 plants) in the same bed (i.e. block). Irrigation, fertilisation and pest control followed standard commercial practices.

Figure 4.2. Strawberry plants were grown in pots filled up with field soil under glasshouse conditions in Experiment (A) 1 and (B) 2. Strawberry plants in Experiment 3 were grown in field plots at (C) one week and (D) 15 weeks post transplantation.

Table 4.2. Background nutrient status analysis[a] (mg kg^{-1}) of the field soil (Barming series) used in the three experiments.

Experiment	pH	NO_3^-	NH_4^+	P	K	Mg
1, 2	5.9	8.1	0.9	31.1	198.6	69.6
3	7.9	2.4	2.0	30.4	188.5	52.1

[a]Growth medium analysis was determined by NRM Laboratories, Berkshire, UK. NO_3^- was determined by ion chromatography and NH_4^+ by colorimetric analysis. P, K and Mg was analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy).

4.1.4 Root sample analysis and plant growth

Plant growth and AMF root colonisation were only assessed at the end of Experiment 1 and 2. Fresh roots were sampled and stained (Section 2.3.1) to assess AMF colonisation (Section 2.3.2). No attempt was made to distinguish between indigenous and pre-inoculated AMF.

The number of plants producing runners, crown size and plant dry weight were only assessed in Experiment 1 and 2. To measure total dry weight, plants were harvested and washed with tap water to remove soil particles. The plants were then oven dried at 80 °C for 3 d and then weighted. To calculate the root weight ratio (RWR: root dry weight as a fraction of the total plant dry weight), roots were weighted after being separated from the shoot. In Experiment 1, nutrient content in strawberry shoot was analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) for P, K, Mg, Ca and micro-elements (Cu, Fe, Zn, B, Mn), while the total nitrogen (N) and sulphur (S) were measured with the Dumas method (AOAC, 1990). Each strawberry shoot sample analysed was pooled from four individual plants from the same block. The shoot nutrient analysis was conducted by NRM Laboratories, Berkshire, UK.

In Experiment 1 and 2, fruit yield was not recorded because plants had not been subjected to adequate chilling and insect pollinators were not provided in the confined compartment (natural pollinators cannot enter the compartment since the facility is completely sealed for the purpose of controlled isolation). In Experiment 3, plant cultivars 'Malling Centenary' and 'Vibrant' (June-bearers) showed an abnormal cropping behaviour (i.e. low flower production) for unknown reasons. Whilst 'Red Glory' (Ever-bearer) flowered normally, the fruits were eaten by birds before harvest. Hence, the fruit yield was also not recorded in Experiment 3.

4.1.5 Disease assessment

Wilt development was scored on the following scale: 0: no wilt symptoms and 1: presence of strawberry wilt symptoms such as wilted leaves, brown leaves, stunted or plant death.

4.1.6 Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). AMF root length colonisation (% RLC) and RWR data were analysed by ANOVA after arcsine square root transformation to satisfy normality. In Experiment 1, there was only one treatment factor (AMF species). In Experiment 2, there were two treatment factors (AMF treatment and cultivar); an unbalanced two-way ANOVA was used to analyse the data. Significant differences among individual treatments were determined by a least significant difference (LSD) post-hoc test if the overall treatment effect was significant ($P \leq 0.05$).

The number of plant producing runners (Experiment 1) and disease incidence (Experiment 3) were analysed using generalised linear models (GLM) with residual errors assumed to follow binomial distributions; the logit link function was used. Only significant differences are reported in the text. For plant growth variables, i.e. crown diameter, RWR and plant dry weight, there were no significant difference among the AMF species tested, thus the data were pooled and treated as single AMF treatment effect. Similarly, the control groups non-AMF control (Cb^-) and non-AMF control with bacterial washing (Cb^+) were not statistically different, thus the data were also pooled and treated as a single non-mycorrhizal (NM) treatment. Therefore, only the overall effect of AMF pre-inoculation on plant growth is presented hereafter.

4.2 Results

4.2.1 Establishment of AMF inoculants after plug transplantation

After cultivation in non-sterilised field soil, microscopic assessment of strawberry roots from Experiment 1 and 2 revealed the presence of AMF structures in the roots of AMF pre-inoculated plants and NM controls. In Experiment 1, at 27 weeks post transplantation all treatments were colonised by AMF to a similar extent (average across all treatments: % RLC reached ca. 93 %; arbuscules and vesicles frequency ca. 32 % and 46 % respectively).

In Experiment 2, average % RLC at 25 weeks reached ca. 90 % across all treatments including the NM controls. There was a significant difference in % RLC between strawberry cultivars 'Vibrant' and 'Red Glory' ($F_{1,18}$ = 6.53; P = 0.020); % RLC was 92 % and 87 % for 'Red Glory' and 'Vibrant', respectively. Arbuscule frequency differed with AMF treatment ($F_{4,18}$ = 3.08; P = 0.043) but not with strawberry cultivar. Plants pre-inoculated with *C. claroideum* had a higher frequency of arbuscules (14 %) than the other AMF treatments (4 %). However, AMF treatment and cultivar did not affect vesicles frequency (average ca. 46 %). There were no significant interactions between AMF treatment and cultivar for any AMF parameter measured.

4.2.2 Effect of AMF pre-inoculation on plant growth

In Experiment 1, all plants survived transplantation and grew normally. The AMF pre-inoculated plants had a tendency to have larger crown size (16.2 ± 0.4 mm; n = 36) than the NM control plants (14.8 ± 0.6 mm; n = 24), although this effect was only weakly significant ($F_{1,56}$ = 3.88; P = 0.054). Plant dry weight and RWR were not influenced by AMF pre-inoculation. However, GLM analysis showed that the production of runners was affected by treatment (P = 0.032) as the number of plants producing runners was less in both the non-AMF control with bacterial washing (Cb^+) and *C. claroideum* treatments than in non-AMF control (Cb^-) (Figure 4.3). Foliar concentrations of both macro-elements (N, S, P, K, Mg and Ca) and micro-elements (Cu, Fe, Zn, B and Mn) were not influenced by AMF pre-inoculation.

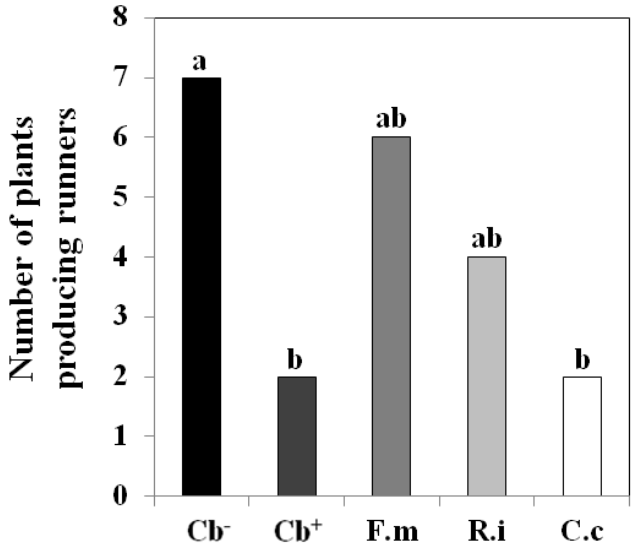

Figure 4.3. Results of generalised linear models fitting number of plants producing runners without pre-inoculated mycorrhiza (non-AMF control (Cb-) and non-AMF control with bacterial washing (Cb+)) or pre-inoculated with single AMF species: *Funneliformis mosseae* (F.m), *Rhizophagus irregularis* (R.i) *and Claroideoglomus claroideum* (C.c) in Experiment 1. Data are number of plants producing runners (n = 12). The overall treatment effect was significant (GLM, P = 0.032). Non-significant differences between treatments are shown by identical letters (Pairwise comparisons, $P \leq 0.05$).

In experiment 2, crown diameter was not affected by AMF pre-inoculation but it was by cultivar ($F_{1,89}$ = 23.1; P < 0.001). Average crown size of 'Vibrant' (15.6 ± 0.3 mm; n = 47) was bigger than 'Red Glory' (13.8 ± 0.3 mm; n = 50). RWR was not affected by AMF pre-inoculation but it was by cultivar ($F_{1,89}$ = 6.6; P = 0.010). Average RWR value of 'Vibrant' (0.42 ± 0.01; n = 47) was higher than 'Red Glory' (0.38 ± 0.01; n = 50). Plant dry weight did not vary with AMF treatment, but the two cultivars differed significantly in total dry weight ($F_{1,89}$ = 49.4; P < 0.001) as 'Vibrant' produced more leaf and root dry biomass (10.6 ± 0.4 g; n = 47) than 'Red Glory' (7.9 ± 0.2 g; n = 50). The interaction between AMF treatment and cultivar was not significant. None of the plants produced runners in Experiment 2.

4.2.3 Effect of AMF pre-inoculation on strawberry wilt incidence

In Experiment 1, 27 weeks after transplantation in soil containing 22.2 CFU g^{-1} of soil, only a few plants showed wilt symptoms (Figure 4.4a-d). In the plants without AMF pre-inoculation two out of 24 plants showed typical wilt symptoms; while in the plants pre-inoculated with AMF, five out of 36 plants were wilted. Such differences were not statistically significant. In Experiment 2, 25 weeks after transplanting in soil containing 22.2 CFU g^{-1} of soil, only a few 'Vibrant' plants showed wilt symptoms (Figure 4.4e-f), whilst all 'Red Glory' plants remained healthy (Table 4.3). The number of diseased plants was too low to allow for meaningful statistical comparison.

Figure 4.4. Strawberry wilt symptoms observed across experiments were characterized by wilted leaves, brown leaves and stunted plants: (a-d) Experiment 1; (e-f) Experiment 2; (g-i) Experiment 3.

Table 4.3. Number of wilted and healthy plants in experiment 2 for treatments without or with AMF pre-inoculation of strawberry cultivars 'Vibrant' and 'Red Glory'

Cultivar	**AMF pre-inoculation**	**Diseased**	**Healthy**
Vibrant	No	2	18
	Yes	3	24
Red Glory	No	0	20
	Yes	0	30

In Experiment 3, 18 weeks after cultivation under field conditions with an average wilt inoculum density of 1.9 CFU g^{-1} of soil, wilt symptoms were observed (Figure 4.4g-i). AMF pre-inoculation increased or decreased the number of diseased plants depending on individual AMF treatment, but differences were not statistically significant. The three cultivars differed significantly in the incidence of wilt according to GLM analysis (P = 0.03). The wilt incidence was in the order of 'Malling Centenary' (158 out of 384) > 'Vibrant' (137 out of 384) > 'Red Glory' (82 out of 384) (Figure 4.5). There were no significant interactions between AMF species and cultivars. The spatial pattern map of strawberry wilt indicated two foci with high numbers of wilted plants (i.e. > 10 diseased plants per plot) (Figure 4.6).

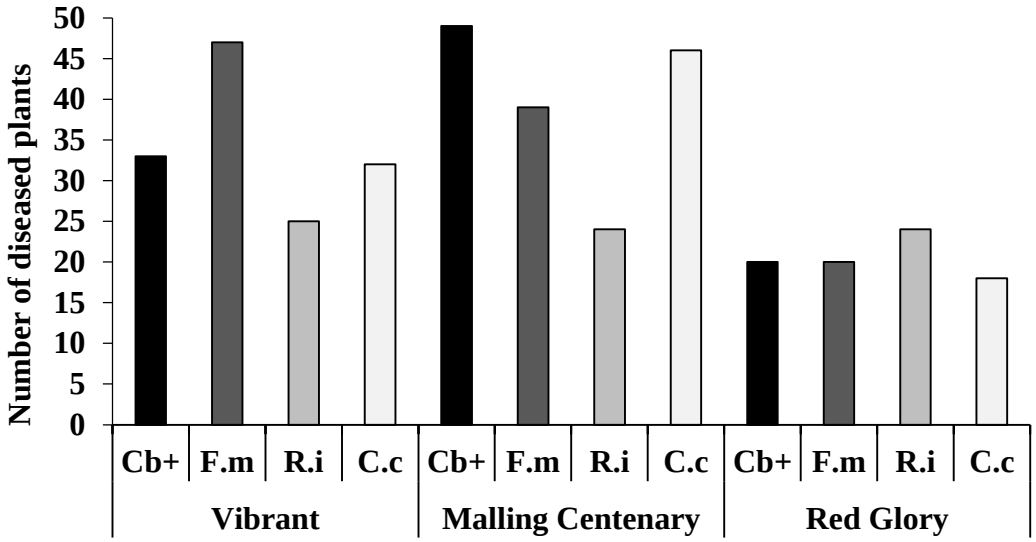

Figure 4.5. Number of diseased plants in experiment 3 without pre-inoculated mycorrhiza (Cb^+) or pre-inoculated with single AMF species (*Funneliformis mosseae* (F.m), *Rhizophagus irregularis* (R.i) and *Claroideoglomus claroideum* (C.c)) of three strawberry cultivars ('Vibrant', 'Malling Centenary' and 'Red Glory'). Data are number of diseased plants (n = 96).

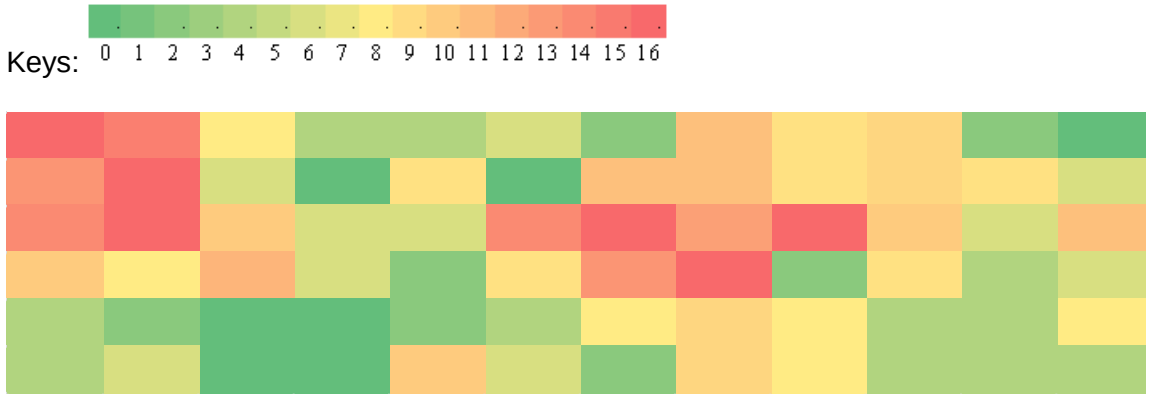

Figure 4.6. Spatial pattern map of *Verticillium dahliae* on strawberry field at 18 weeks post transplantation. Legend bar represent the colour key associated with the number of diseased plants per plot (value). There were six blocks (i.e. six planting beds), 72 plots with 16 plants each (AMF treatment and strawberry cultivar for each individual plot are not presented for clarity

4.3 Discussion

Since AMF have been shown to have positive effects on strawberry growth and health (Khanizadeh et al. 1995, Norman et al. 1996a, Tahmatsidou et al. 2006, Sowik et al. 2016), there is an increasing interest to incorporate them into production practices to increase yield and fruit quality while reducing fertiliser and biocides inputs. After several months of culture in pots filled up with field soil, AMF colonisation of strawberry roots was high in Experiment 1 and 2. These results agree with previous studies showing that strawberry plants can be heavily colonised by AMF in soil (Robertson et al. 1988, Vestberg et al. 2000, Santos-González et al. 2011). At the end of Experiment 1 and 2, the controls and AMF pre-inoculated treatments showed similar levels of AMF root colonisation. This observation confirmed the presence of indigenous AMF in the field soil used for the pot experiments. In addition, 'Vibrant' showed overall a lower AMF root colonisation level than 'Red glory' in Experiment 2. This is in agreement with other studies showing that strawberry cultivars could differ in their response to AMF colonisation under both glasshouse or field conditions (Robertson et al. 1988, Chávez and Ferrera-Cerrato 1990, Vestberg 1992c, Khanizadeh et al. 1995). Moreover, it was not possible to confirm whether pre-inoculated AMF species persisted in the pot experiments due to the presence of AMF root colonisation in the controls. A number of studies have already discussed the fact that soils containing native AMF propagules are problematic in the sense that inoculated fungi cannot be distinguished visually from the indigenous AMF (Niemi and Vestberg 1992, Tahmatsidou et al. 2006, Rodriguez and Sanders 2015). Therefore, metagenomic approach may be used in the future to assess whether pre-inoculated AMF species persisted temporally and spatially and if they altered the composition of the native AMF community in the field soil (Rodriguez and Sanders 2015).

Pre-colonisation by AMF did not result as expected in an enhanced plant growth (e.g. plant dry biomass) after transplantation in pots containing non-sterile field soils. It was suggested that in such long-term experiments the indigenous AMF inocula present in the soil used for this work could have masked the initial positive effect on plant growth provided by AMF pre-colonisation. Nevertheless, future experiments with other strawberry cultivars and sampling times closer to the transplantation time may reveal whether the pre-inoculation was of any advantage for plant growth and nutrition in the early stage of establishment in the field or sandy compost. Moreover, non-mycorrhizal control plants inoculated with bacterial filtrate in Experiment 1 indicated a negative effect of the background bacterial community of the commercial AMF inoculants on the number of plants producing runners. Several authors have discussed the potential of some bacterial strains to positively influence the production and quality of strawberry runners (Aslantaş and Güleryüz 2004, Pirlak and Köse 2010), but this the

first report of a negative effect of bacteria associated with the AMF inoculants on the production of strawberry runners. Therefore, additional work is needed to confirm the results and identify candidate microbes responsible for this phenomenon.

Furthermore, it was not possible to confirm that early AMF colonisation can improve plant tolerance to wilt under glasshouse conditions (Experiment 1 and 2). In fact, natural *Verticillium* inoculum (microsclerotia) only resulted in a very low level of wilt symptoms despite the high level of viable microsclerotia in the soil (22 CFU g^{-1} of soil) that should have induced wilt symptoms to the susceptible strawberry cultivar tested. The reasons for such result are unknown. Furthermore, drenching soil with wilt conidial suspension without root injury in sandy compost or hyphae inoculations with colonised potato dextrose agar (PDA) media plates buried at the bottom of the pots filled up with attapulgite clay failed to induce wilt symptoms (data not presented). Hence, it is of great importance to select very susceptible strawberry cultivars (e.g. 'Elsanta', 'Emily'), more virulent wilt strains and/or optimise another wilt inoculation method to enable further research under controlled conditions. Root-dipping inoculation with artificial root injury (Bhat and Subbarao 1999) can be proposed as potential solution to obtain more disease symptoms under pot conditions (Fan & Cockerton, pers. comm.). This method however was not here adopted in order to avoid potential disruption or damage to injure the established mycorrhizal network in the strawberry plant plug.

Nonetheless, the results of pot experiments cannot be directly compared to open field conditions, where much more complex system of animals, microbes and nutrients prevail. When the field soil was transferred into pots its structure was dramatically modified (e.g. by sieving) and glasshouse conditions were rather different from those in the open field. In the current study, these differences may have modified complex interactions between plants, soil borne pathogens and/or beneficial microbes resulting in the low number of wilted plants observed despite the high wilt propagule density in the soil.

In the open field study (Experiment 3), all three susceptible strawberry cultivars tested suffered from *Verticillium* wilt. The typical strawberry wilt symptoms developed across plots contaminated in average with 1.9 CFU g^{-1} of soil. However, results showed that none of the pre-inoculated AMF species could significantly reduce wilt incidence under open field conditions. Although AMF inoculants were not effective under the conditions tested, this does not rule out their usefulness with other strawberry cultivars and/or in other locations. In addition, the spatial pattern of the *Verticillium* wilt indicated a high degree of aggregation of diseased plants, which was also observed with *V. dahliae* on other crops (Xiao et al. 1997, Johnson et al. 2006, Wei et al. 2015). Therefore, soil samples from each experimental plot should have been tested for wilt propagule concentration (e.g. via wet-sieving plating or wet-sieving qPCR method) before strawberry plug transplantation to confirm whether higher CFU values led to

increase wilt incidence in those areas and/or to correct for spatial aggregation of wilt propagules on treatment effects during statistical analysis. However, accurate testing of wilt soil inoculum is very time consuming and impractical for every plot, whilst the newly developed qPCR tests (by Fera and ADAS) is not sufficiently sensitive.

5 Can an axenic autotrophic *in vitro* system be used to explore the nature of interactions between AMF and soil-borne pathogens in strawberry?

5.1 Introduction

Among other benefits, arbuscular mycorrhiza fungi (AMF) colonisation is known to alter strawberry plant response to biotic stresses, leading to increased tolerance to attacks by root pathogens (Norman et al. 1996a, Tahmatsidou et al. 2006, Sowik et al. 2016). However, the interactions among AMF and pathogens are complex; a further complicating factor is most of these studies have been conducted under glasshouse or field conditions where changes in the environment also affect the nature of these interactions. Therefore, investigating the nature of the interaction between pathogens and AMF under *in vitro* conditions may help to reveal the role of AMF in increased tolerance/resistance to strawberry soil-borne pathogens.

The application of autotrophic systems (i.e. *in vitro* culture systems with photosynthetic active plant tissues) may therefore have been described as a useful approach to dissect various aspects of plant–AMF interactions. The AMF symbiosis has been successfully established under axenic or semi-axenic controlled conditions with several plant species (e.g. banana, barrelclover, clover, ficus, potato and vine; (Hepper 1981, Voets et al. 2005, Koffi et al. 2009, Voets et al. 2009, Nogales et al. 2010, Lovato et al. 2014) including strawberry (Elmeskaoui et al. 1995, Cassells et al. 1996). Autotrophic systems may therefore facilitate the study of biochemistry, molecular and physiological aspects of the plant–AMF–pathogen interaction, allowing more accurate assessments than in conventional pot systems. Recently, several autotrophic systems were developed to study the effect of AMF association against pathogens and demonstrated the protective effect of AMF (Nogales et al. 2010, Koffi et al. 2013, Lovato et al. 2014, Oye Anda et al. 2015). In addition, the *in vitro* system developed by Sowik *et al.* (2008) allowed the successful association of strawberry plantlets with *Verticillium dahliae* under axenic conditions, speeding up screening for wilt resistance. Although strawberry roots were successfully associated with AMF under *in vitro* conditions (Elmeskaoui et al. 1995, Nuutila et al. 1995, Cassells et al. 1996), interaction studies between the plant, AMF and pathogens have not been reported yet under axenic conditions.

This study aimed to study protective effects of AMF against *V. dahliae* or *P. fragariae* under an axenic *in vitro* culture system by adapting autotrophic culture systems developed by Müller *et al.* (2013) and Voets *et al.* (2009). *Rhizophagus irregularis* (MUCL 43194) was used as the AMF inoculant and two strawberry pathogens were tested separately: *V. dahliae* was inoculated onto *Fragaria vesca* (one diploid parent of cultivated strawberry) and *Phytophthora fragariae* (red core) was inoculated onto *F. ananassa* cv. ‘Calypso’. Mycorrhiza spore

germination, spread of the pathogens on the medium and intraradical root colonisation by AMF and the pathogens were assessed.

5.2 Materials and methods

Two experiments were carried out and a schematic representation of the experimental setup is shown in Figure 5.1.

5.2.1 Plant materials and arbuscular mycorrhiza fungus

Tissue culture plants of an *F. vesca* clone VSI and *F.* x *ananassa* cv. 'Calypso' produced as discribed in Section 2.2.1 were used in both experiments. *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessler 2010, strain MUCL 43194, was purchased from Premier Tech Biotechnologies (Mycorise® ASP, Rivière-du-Loup, Canada) as a sterile suspension of spores (400 spores mL^{-1}) and used to inoculate strawberry plants.

5.2.3 Strawberry plantlets establishment in axenic conditions

The autotrophic *in vitro* system was adapted from axenic culture systems developed by Müller *et al.* (2013) and Voets *et al.* (2009). Square Petri dishes (12 x 12 cm) were filled with Modified Strullu Romand (MSR) medium (Declerck et al. 1998), lacking sucrose and vitamins, and solidified with 5 g L^{-1} Phytogel™. After solidification, the upper half of the medium was removed from the Petri dishes. Two microplants were then transferred into each square plate, with the roots placed on the surface of the MSR medium and the shoot extending in the empty part of the plate. The square plates were then sealed with parafilm and incubated vertically in a growth room (21 °C, light:dark 16 h/ 8 h, photosynthetically active radiation (PAR) of 40 µmol m^{-2} s^{-1}) for 3 weeks to allow plants to produce new roots.

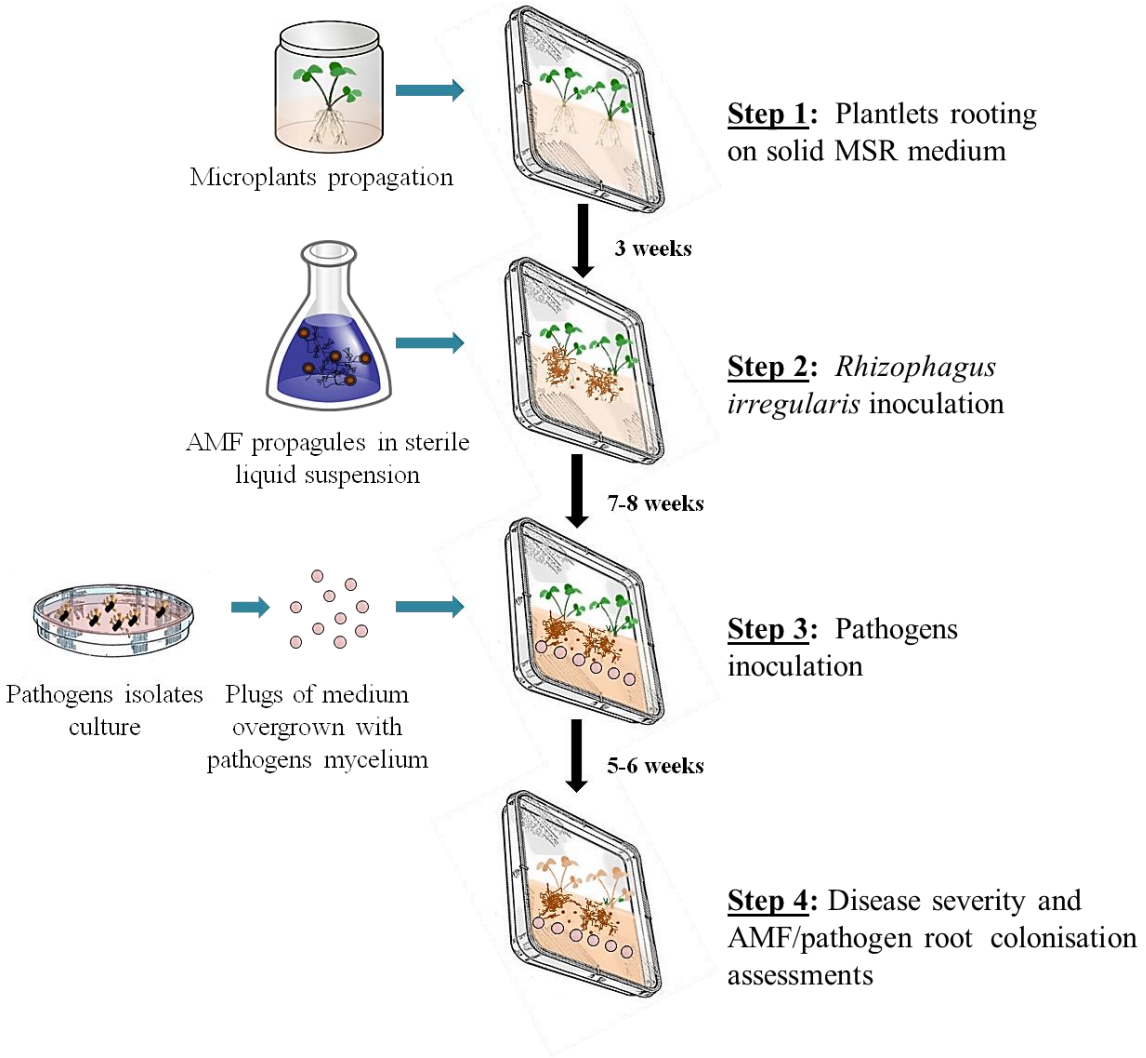

Figure 5.1. A schematic representation of the experimental setup to study the interaction of arbuscular mycorrhiza fungi (AMF) and *Verticillium dahliae* (wilt) or *Phytophthora fragariae* under *in vitro* culture: (1) strawberry microplants were rooted on modified Strullu Romand (MSR) medium; (2) microplants were then inoculated with commercial sterile *Rhizophagus irregularis* spores; (3) microplants were inoculated with plugs of medium overgrown with pathogen mycelium and (4) disease severity in addition to AMF or pathogen root colonisation was assessed.

5.2.4 Strawberry plantlets inoculation with AMF

After 3 weeks on the MSR medium, each plant was inoculated with 250 µL (ca. 100 spores) of the sterile *R. irregularis* spore suspension. For each experment, 20 square plates with AMF (+AMF treatment) and 20 without AMF (-AMF treatment) were set up. The plates were sealed again with parafilm and the root area was covered with alluminum foil to allow the roots and AMF to grow under darkened conditions. The square plates were incubated for 7-8 weeks after they were inoculated with AMF.

5.2.5 Soil-borne pathogens

Three isolates of both *V. dahliae* and *P. fragariae* pathogens from the collection held at East Malling Research (EMR), Kent, UK were used in this study.

For the strawberry wilt inoculation (Experiment 1), three *V. dahliae* isolates (12251, 12252 and 12253) were recovered from EMR cryostore and cultured separately on a sterile prune lactose yeast agar (PLYA) medium (Talboys 1960) for ca. 1 month in the dark at 22 ± 1 °C. Five mL of sterile purified water was pipetted onto each Petri dish and the medium surface gently rubbed to make a conidial suspension. Finally, 0.2 mL conidial suspension of each isolate was pipetted onto the same fresh PYLA plate; the plates were then incubated for 18 d at ambient conditions close to a natural light source.

For the red core inoculation (Experiment 2), three *P. fragariae* isolates (BC-1, BC-16 and Nov-9) were cultured separately on sterile modified kidney bean agar (KBA) medium (Maas 1972) for 1-2 months in the dark at 18 ± 1 °C. Then one piece (1-4 mm^2) of colonised KBA (from stock culture) was transferred to a fresh KBA medium plate, and then incubated in the dark at 18 ± 1 °C for ca. 3 weeks.

5.2.6 Inoculation of strawberry plantlets with pathogens

In Experiment 1, after 8 weeks of growth in plates with or without AMF, the plantlets were randomly divided in four treatments: inoculated with (1) wilt and AMF (+AMF+Wilt), (2) AMF only (+AMF-Wilt), (3) wilt only (-AMF+Wilt), and (4) no AMF or wilt (-AMF-Wilt). Each treatment had ten replicates each with two plantlets. Inoculation with *V. dahlia* was as follows: hyphal plugs, 5 mm in diameter, were harvested from 18 d old colonised PLYA Petri dishes using a sterilised cork borer and transferred onto the roots (three plugs per plant) (Figure 5.4A). The square plates were then sealed, shaded with aluminium foil and incubated in a growth room (Meridian Refrigeration Ltd, Croydon, UK; day and night 21-22 °C, light: dark 16 h/8 h, PAR of 37-42 µmol m^{-2} s^{-1}). The position of each plate in the growth room was randomised.

In Experiment 2, after 7 weeks of growth, the plantlets were randomly divided in four treatments: inoculated with (1) *P. fragariae* and AMF (+AMF+Pf), (2) AMF only (+AMF-Pf), (3) *P. fragariae* only (-AMF+Pf), and (4) no AMF or *P. fragariae* (-AMF-Pf). Plantlets of +AMF+Pf and -AMF+Pf treatments were then inoculated with *P. fragariae.* Inoculation with *P. fragariae* was as follow: Hyphal plugs, 5 mm in diameter, were cut from the growing edge of 3 weeks old colonised KBA Petri dishes and transferred onto the plants roots (one plug from each of the three isolates per plant). The plates were sealed, covered with foil, and incubated in a growth room (Meridian Refrigeration Ltd, Croydon, UK, day and night 15-16 °C, light: dark 16 h/8 h, PAR of 40 µmol m^{-2} s^{-1}) (Figure 5.2); the position of each plate in the growth room was randomised.

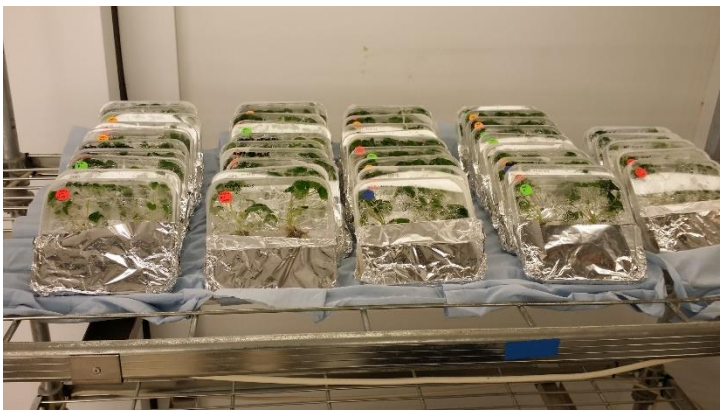

Figure 5.2. A photo depicting the square plates of Experiment 2 incubated in a growth room at 15 °C after inoculations of plantlets with *Phytophthora fragariae* hyphal plugs.

5.2.7 Estimation of disease severity and assessment of root colonisation by AMF and pathogens

In both experiments, disease severity was assessed 5 week after inoculation with hyphal plugs. The symptom was assessed on a rating scale from 0 to 5: 0 - no symptoms, 1 - shoot with a single leaf showing symptoms (yellowish-brown appearance), 2 - up to 25 % of leaves showing symptoms, 3 - up to 50 % of leaves showing symptoms, 4– up to 75 % of leaves showing symptoms, and 5 - complete plant death.

After the disease severity assessment, AMF root colonisation (i.e. hyphae, arbuscules and vesicles) was assessed on the plantlets in the mycorrhizal treatments (+AMF). Similarly pathogen structures (i.e. hyphae and microsclerotia for *V. dahliae* and oospores for *P. fragariae*) were assessed in those plantlets inoculated with the pathogens. The plantlets roots were carefully removed from the square plates with a pair of forceps and stained as described in Section 2.3.1. Each root sample was pooled from four individual plantlets (i.e. two square plates).

5.2.9 Data analysis

All data were analysed using R-3.0.2 with the R-Studio interface. The disease severity data were analysed using the Mann-Whitney *U* test. In both experiments, there was no AMF colonisation in the mycorrhizal treatments (+AMF). Therefore, to analyse the disease severity, data from the mycorrhizal treatments were pooled with their respective non-mycorrhizal treatments.

5.3 Results

Fragaria vesca and *F.* x *ananassa* cv. ‘Calypso’ plantlets were able to produce new roots and leaves on modified MSR medium under completely sealed environment (Figure 5.3A, B). In addition, *R. irregularis* (MUCL 43194) could germinate on modified MSR medium (Figure 5.4A,

B), but no AMF intraradical colonisation was observed after trypan blue staining of *F.* x *ananassa* and *F. vesca* roots. The mycelium of both pathogens was able to grow and spread on MSR medium in the presence of plant roots (Figure 5.5A, B). Staining of *F. vesca* roots with trypan blue failed to detect wilt infection (i.e. presence of hyphae and microsclerotia). Nevertheless, the shoot showed symptoms of wilt (Figure 5.6A) and there was a difference in disease severity between the *V. dahliae* inoculated (+Wilt) and non-inoculated treatment (-Wilt) (Mann- Whitney *U* test, $P = 0.002$). The median severity score was 2.75 for plantlets without *V. dahliae* (-Wilt) and 3.75 for plantlets with *V. dahliae* (+Wilt). *F.* x *ananassa* cv. 'Calypso' plantlets were highly infected by *P. fragariae* (Figure 5.6B) with the presence of abundant oospore in the root tissues (Figure 5.5C) and the petiole of the leaves (Figure 5.6D). There was a significant difference in the disease severity between the *P. fragariae* inoculated (+Pf) and non-inoculated treatment (-Pf) (Mann- Whitney *U* test, $P < 0.001$). The median severity score was 1 for plantlets without *P. fragariae* (-Pf) and 5 for pathogen inoculated plantlets (+Pf).

Figure 5.3. (A) *Fragaria. vesca* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets with well-established root systems and healthy shoots after one month of culture on modified Strullu Romand (MSR) medium under axenic conditions.

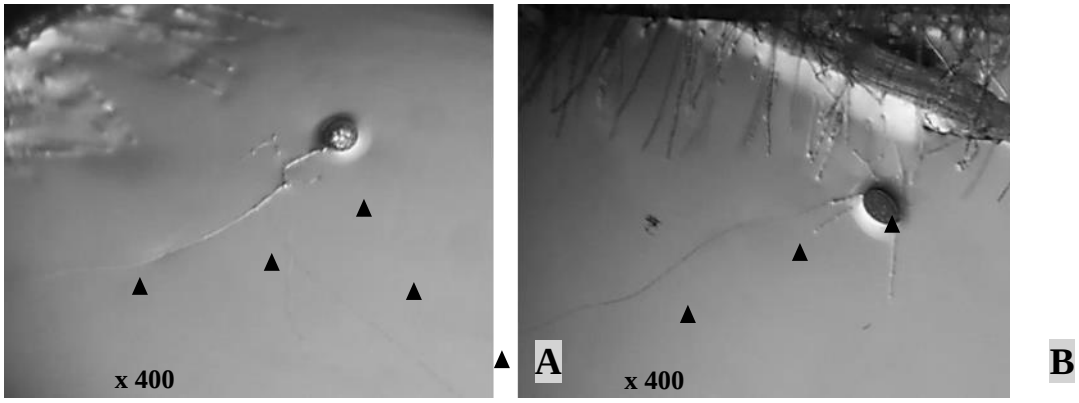

Figure 5.4. Germinated *Rhizophagus irregularis* spores (black arrow = mycorrhizal hyphae) on modified Strullu Romand (MSR) medium. Photos were taken after two months of culture under axenic conditions with (A) *Fragaria vesca* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets. The scale bar feature was not available in the camera used for image acquisition thus the

magnification is reported instead.

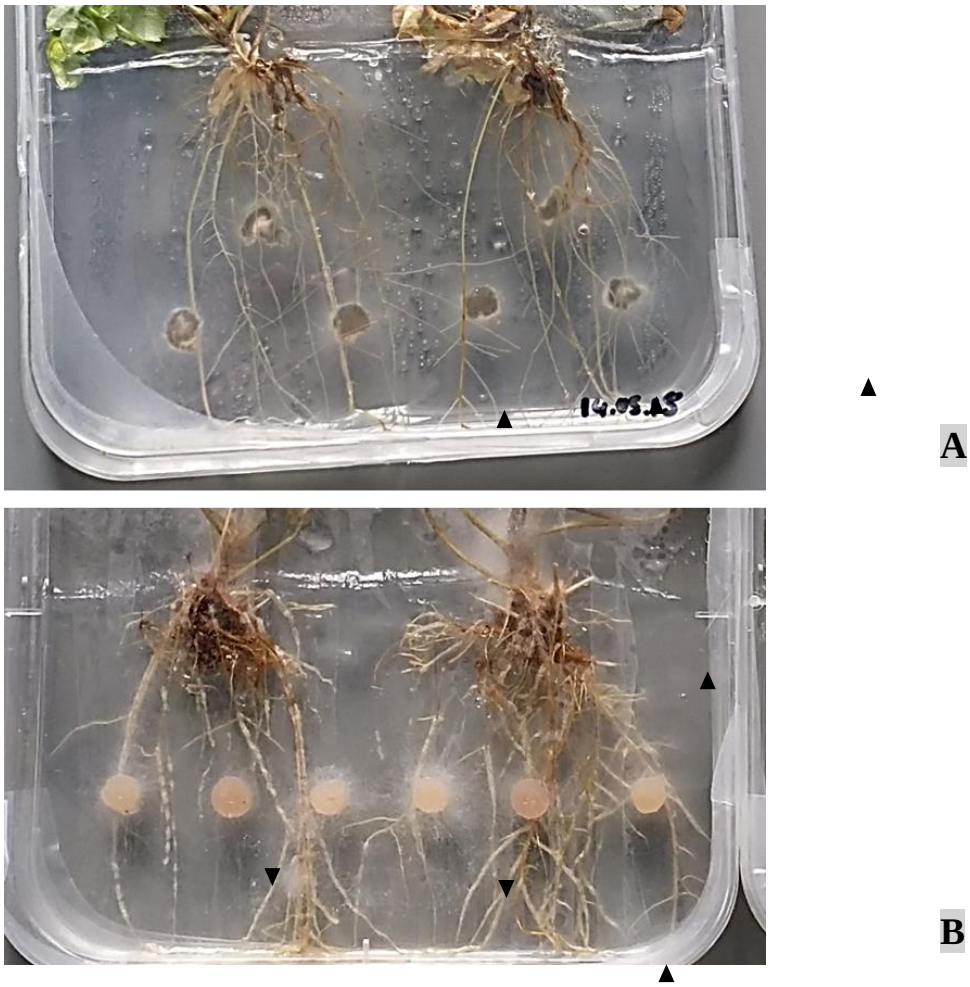

Figure 5.5. (A) *Fragaria vesca* plantlets inoculated with hyphal plugs of *Verticillium dahliae* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets inoculated with hyphal plugs of *Phytophthora fragariae* 5 weeks after pathogen inoculation. Both pathogens could spread and establish on the surface of modified Strullu Romand (MSR) medium (black arrows highlight areas where the mycelium grew clearly around the mycelial plugs).

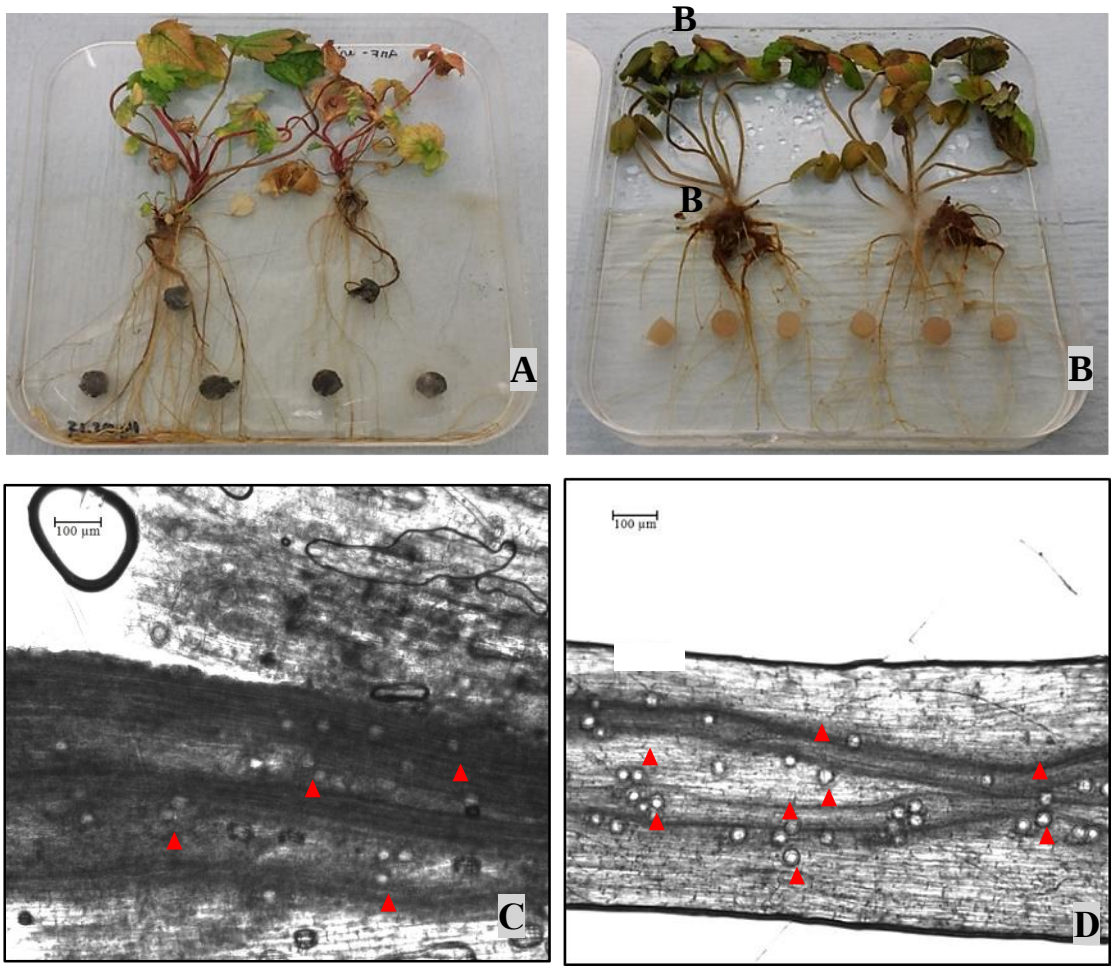

Figure 5.6. Disease symptoms of (A) *Fragaria vesca* plantlets inoculated with *Verticillium dahliae* and (B) *F.* x *ananassa* cv. 'Calypso' plantlets inoculated with *Phytophthora fragariae*, 5 weeks after pathogen inoculation. Longitudinal squash of 'Calypso' (C) leave petiole and (D) root stained with trypan blue showing the presence of *P. fragariae* oospores (red arrows). The black scale bars represent 100 µm.

5.4 Discussion

In this study, both *V. dahlia* or *P. fragariae* could induce disease symptoms on strawberry plantlets on MSR medium *in vitro*. However, the root colonisation by *V. dahlia* could not be confirmed by optical microscopic observations. It is possible that the pathogen did not establish within the plant roots. Therefore, in future experiments other staining or detection methods could be tested to confirm the presence of strawberry wilt in the root tissues. For example, the use of WGA-AF488 / Propidium Iodide has been suggested to be suitable for the staining of *V. dahliae* within plant roots (Liang, 2012; Taylor pers. comm.), alternatively a PCR bioassay could be used to confirm the presence of wilt DNA within the root tissues (Mirmajlessi *et al*., 2015). In addition, the presence of plants with yellow or brown leaves was also observed, but to a lesser extent, in culture plates without pathogens in both experiments. This observation

may be the result of natural aging and/or nutrient depletion of the growing medium. Therefore, to try and improve the culture system and the precision of disease severity assessments, fresh MSR medium could be added to the square plates (Voets *et al.*, 2009; Oye Anda *et al*., 2015).

Unfortunately, the potential protective effect of AMF against *V. dahliae* or *P. fragariae* could not be verified using the current culture system as AMF colonisation was not achieved. The reasons for this however are unknown as the sterile spores of *R. irregularis* were viable as observed by their germination on the MSR medium in both experiments. In parallel with the *in vitro* system, *R. irregularis* spores were inoculated onto microplants of *F. vesca* and *F.* x *ananassa* cv. 'Calypso' during the weaning stage on attapulgite clay and were able to colonise plant roots (data not shown). In the context of *in vitro* culture conditions, several factors may have disturbed the interaction between the hosts and AMF. For example, the limited gas exchange in this completely sealed *in vitro* environment may have been responsible for high relative humidity that may lower photosynthetic photon flux density (PPFD) due to water condensation (see Figure 5.3A), harmful gas accumulation and/or depletion of CO_2 concentration (Liu and Yang, 2008). Substrate conditions such as inadequate pH, low porosity and/or high thickness could also have altered AMF hyphal development and function (Mosse 1988, Liu and Yang 2008, Costa et al. 2013).

Several culture systems may be able to overcome the problem of gas exchange, water condensation and lack of light. The adaptation to strawberry plantlets of the semi-axenic systems developed by Voets *et al*. (2005) and improved by Koffi *et al*. (2009) appear to be an interesting solution. The aim of this study was to develop a simple experimental culture system associating autotrophic micropropagated strawberry plantlets with AMF and several soil-borne pathogens in an axenic environment. For this reason, the semi-axenic system was not adopted in this study. In fact semi-axenic systems are more complex than axenic ones (i.e. additional steps are required), it is more sensitive to contamination due to additional interventions on the growing plates (i.e. small opening on the plate lid) and there is a risk of microplant death by desiccation at acclimatisation. Nevertheless, in semi-axenic systems, because the shoots of the plantlets can grow outside of the culture plate, the risk of high relative humidity, harmful gas accumulation and/or depletion of CO_2 will be absent. In addition, a modified mycelium donor plant (MDP) *in vitro* culture system may allow for a faster and homogenous colonisation of strawberry plantlets. Voets *et al*., (2009) developed this semi-axenic system and they showed a successful AMF colonisation of *Medicago truncatula* roots transferred on already actively growing extraradical hyphae extending from *M. truncatula* donor plants.

6 Evaluating the effects of arbuscular mycorrhiza fungi and plant-growth promoting rhizobacteria on strawberry productivity and tolerance to *Phytophthora* diseases in substrate

6.1 Introduction

Control of soil-borne pathogens is a major problem in strawberry field production since the withdrawal of the effective broad-spectrum chemical fumigant methyl bromide (Ristaino and Thomas 1997, Tahmatsidou et al. 2006). Recently, UK strawberry production has rapidly moved away from traditional field cultivation towards table-top system, where strawberry plants are grown in soil-less substrate (e.g. coir and/or peat) under protection (Porter and Mattner 2002, Boyer et al. 2016). There are several advantages to the adoption of soil-less substrates in commercial strawberry cropping, including reduction in harvest costs, extension of the growing season and reduced damages caused by soil-borne pathogens (Paranjpe et al. 2008, Martínez et al. 2010, Lieten 2013). Nevertheless, *Phytophthora fragariae* (red core) and *P. cactorum* (crown rot) continue to pose a serious threat to strawberry growing in soil-less substrate (Schnitzler 2004, Martínez et al. 2010) as they may have infected initial planting materials in nurseries.

By inoculating the strawberry rhizosphere with beneficial microbes, plants could be potentially be protected against biotic (e.g. pathogens) and abiotic (e.g. drought) stresses, while water and nutrient uptake could also be improved (Vestberg et al. 2004a, Boyer et al. 2016). Strawberry is an ideal production system to study such methods as planting materials (e.g. micro-propagated or runners derived plug plants) can be easily inoculated with beneficial microbes during their propagation and/or at planting. Several biological inoculants have already demonstrated the potential to reduce the threat of strawberry root diseases in soil-less substrates when introduced at planting (Martinez et al. 2013, Rouphael et al. 2015). Amongst those beneficial microbes, arbuscular mycorrhizal fungi (AMF) is shown to increase strawberry yield and tolerance to drought in soil-less substrates (Cekic and Yilmaz 2011, Boyer et al. 2014, Palencia et al. 2015, Boyer et al. 2016, Cecatto et al. 2016) and/or increased tolerance to root pathogens such as *P. cactorum* and *P. fragariae* (Murphy et al. 2000a, Vestberg et al. 2004b). In addition, plant growth-promoting rhizobacteria (PGPR) have the potential to produce substances that can promote plant growth and/or protect plant against root pathogens (Glick 1995, Vestberg et al. 2004b). Several studies have highlighted beneficial effects of PGPR on strawberry health and/or productivity (Esitken et al. 2010, Ipek et al. 2014, Hautsalo et al. 2016). Interestingly, synergistic effects on strawberry growth following co-inoculation with PGPR and AMF were reported (Vosatka et al. 1992) and PGPR were also found to stimulate AMF root colonisation (Vosatka et al. 2000).

The first aim was to investigate whether commercially available AMF and/or PGPR inoculants can reduce *P. cactorum* or *P. fragariae* development in soil-less substrates. Strawberry cultivars 'Malling Centenary' or 'Vibrant' were used to test the hypothesis that AMF pre-inoculation and/or inoculation of AMF and/or PGPR at planting time can enhance tolerance to *P. fragariae* in sandy compost. Then 'Malling Centenary' was used to study whether AMF pre-inoculation can increase tolerance to *P. cactorum*. The second objective was to assess whether commercially available AMF and PGPR inoculants can increase strawberry yield in coir. Strawberry cultivar 'Malling Centenary' was used to test the hypothesis that inoculation of AMF and/or PGPR at planting time can increase strawberry yield.

The experiments in this section were conducted jointly with SF157 (integrated strawberry disease management) where Joyce Robinson and Tom Passey help to conduct the experiments funded by SF157 (hence Phytophthora results were also presented in the SF157 report).

6.2 Materials and methods

A total of four experiments were conducted in 2015-2016. Experiment 1 and 2 were carried out to study the protective effect of AMF and/or PGPR against *P. fragariae* under growth room conditions. Experiment 3 was carried out in a glasshouse compartment to test whether AMF can control *P. cactorum*. Finally, experiment 4 was conducted under glasshouse conditions to evaluate the effect of AMF and/or PGPR on strawberry plant growth and productivity in coir. Table 6.1 gives the summary of each experiment.

6.2.1 Plant material

Cold stored (-2°C) strawberry plugs used in Experiment 1, 3 and 4 (Table 6.1) were obtained from the same commercial nursery (Hargreaves Plants, Norfolk, UK); plants derived in this way have shown in previous work to be free from AMF colonisation (Xu, unpublished data) and a number of plants were tested prior to the main experiments to confirm this.

For Experiment 2, AMF pre-inoculated and non-mycorrhizal strawberry plugs were obtained from a commercial nursery (R W Walpole Ltd, Norfolk, UK). Plastic trays (40 cells, ca. 132 cm^3 per cell; PG Horticulture Ltd, Northampton, UK) were filled up with a peat/perlite/coir mix (7:2:1, v/v; fertilised by the supplier with Osmocote® [15N-9P_2O_5-11K added at 3 kg m^3] and Micromax® premium [trace elements and magnesium fertiliser added at 0.3 kg m^3]; Legro Beheer b.v., Asten, The Netherlands) using an automatic Javo tray filler (Javo b.v., Noordwijkerhout, The Netherlands; Figure 6.1A-C). For the AMF inoculated plants, Rootgrow™ (PlantWorks Ltd, Kent, UK) was mixed homogeneously with the potting mix at the ratio of 10 % (v/v), while only the potting mix was used for the non-mycorrhizal treatment. On 7th July 2015 uniform runner tips of cv. 'Malling Centenary' (propagated by R W Walpole Ltd, Norfolk, UK) were harvested and then pinned-down under glasshouse conditions. The plantlets were firstly weaned (using a misting system spraying water every hour for the first 5 d aiming for relative humidity (RH) 80 % and then every 3 hours for the next 5 d, while plants were protected from direct sunlight; Figure 6.1D). Then misting system was switch off. The plants were irrigated as needed, no additional fertiliser was added and direct sunlight was progressively introduced. The plug plants were grown for seven weeks (Figure 6.1E). On 27th August 2015, plug plants were sent to EMR, where they were cold stored at 2 °C for 14 weeks. A few plants were tested prior the experiment to confirm whether the pre-inoculation was successful (i.e. 10 samples, each with five plants), which indicated the presence of a low level of mycorrhizal structures in root (Figure 6.2) with average % RLC of ca. 3 %.

Table 6.1. Summary of the four experiments to study the effect of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) on strawberry plant health or productivity in soil-less substrates under controlled conditions.				
Microbial treatment	**Experiments**			
	1	**2**	**3**	**4**
Number of treatments	4	8	2	4
Treatments[a]	M, P, M+P, Cb^-	M, P, M+P, PreM, PreM+M, PreM+P, PreM+M+P, Cb^-	M, Cb^-	M, P, M+P, Cb^-
Plant cultivars	'Vibrant'	'Malling Centenary'	'Malling Centenary'	'Malling Centenary'
Total no. of replicates[b]	20	16	20	3
No. blocks	4	4	-	-
No. of repeated experiments	1	1	2	2
Storage temperature of plugs	-2 °C	+2 °C	-2 °C	-2 °C
Location	Growth room	Growth room	Glasshouse (chilled)	Glasshouse
Growing substrate	Sandy compost	Sandy compost	Compost	Coir
Pathogen inoculated	*P. fragariae*	*P. fragariae*	*P. cactorum*	-
Pathogen inoculation method	Slurry	Slurry	Wound + zoospores	-
Pathogens concentration[c]	-	-	10^3 CFU mL^{-1}	-
Planting (AMF inoculation) date	20/07/15	18/12/16	17/06/16 (repeat 1) 20/06/16 (repeat 2)	17/05/16 (repeat 1) 24/05/16 (repeat 2)
Experimental duration	5 weeks	6 weeks	7 weeks	13 weeks

[a] **M**: commercial AMF mix inoculum (Rootgrow$^{TM)}$ containing five species, *Funneliformis mosseae*, *F. geosporum Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*, (disclosed by PlantWorks Ltd, Kent, UK) inoculated at planting. **P**: bacterial preparation containing four PGPR species, *Rhizobium* (Agrobacterium) strain IRBG74, *Derxia lacustris* HL-12, *Bacillus megaterium* and *B. amyloliquefaciens*

(disclosed by PlantWorks Ltd, Kent, UK) inoculated at planting. **M+P**: AMF and PGPR co-inoculated at planting. **PreM**: plug plants pre-inoculated with Rootgrow^{TM} during tipping. **PreM+M**: AMF pre-inoculated plugs inoculated with Rootgrow^{TM} at planting. PreM+P: AMF pre-inoculated plugs were inoculated with PGPR at planting. **PreM+M+P**: AMF pre-inoculated plugs were inoculated with Rootgrow^{TM} and PGPR at planting. **Cb^{-}**: negative control without AMF pre-inoculation and no microbial inoculum added at planting.

[b] In Experiment 4, each coir bag (replicate) was planted with 10 strawberry plants.

[c] CFU stands for colony-forming unit.

Figure 6.1. (A) Bale breaker loading the tray filler machine with potting substrate (i.e. peat/perlite/coir mix: 7:2:1, v/v), here inoculated with the commercial AMF inoculum Rootgrow™. (B) Javo tray filler loading the plastic trays with potting mix. (C) View of a plastic tray filled with potting mix. (D) Strawberry plugs weaned under a misting system. (E) Plug plants grown under glasshouse conditions seven weeks post transplantation.

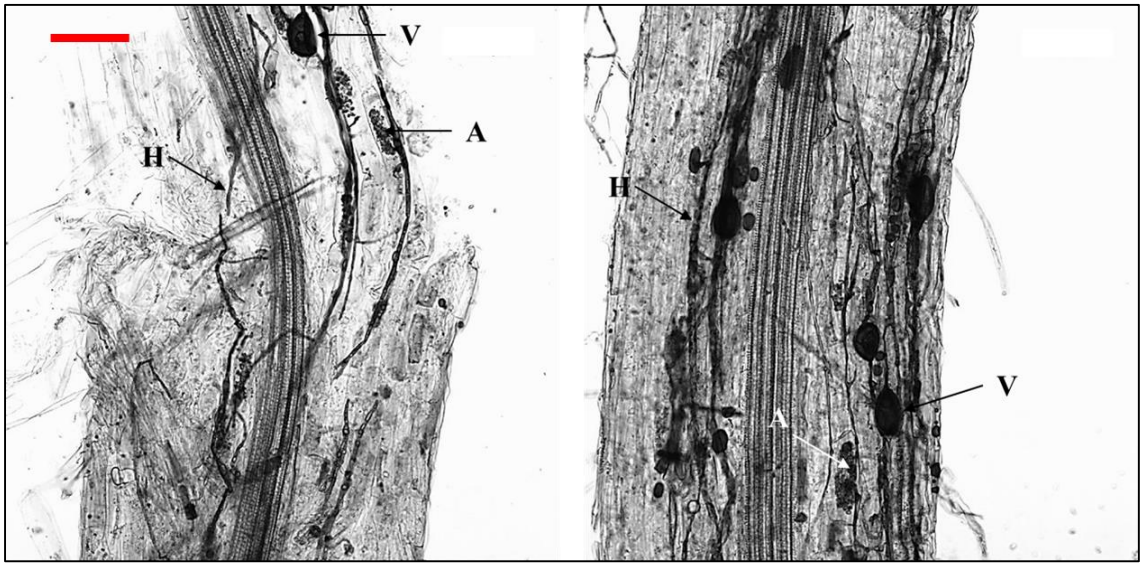

Figure 6.2. Root colonisation by arbuscular mycorrhiza fungi (AMF) of strawberry plugs in Experiment 2 after 21 weeks (i.e. after 7 weeks under glasshouse conditions and 14 weeks in a dark storage compartment at 2°C) of cultivation. Letters next to the arrows are A: arbuscule, H: hyphae, V: vesicle (red scale bars represent 100 µm).

6.2.2 Beneficial microbe inoculations

Inoculum of AMF and PGPR were provided by PlantWorks Ltd, Kent, UK. The AMF granular formulation was applied as commercially available Rootgrow^{TM}, a clay/pumice/zeolite mix containing spores, mycelium, and colonised host plants root fragments of five different AMF species (*Funneliformis mosseae*, *F. geosporum Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Glomus microagregatum*). Rootgrow^{TM} contained ca. 350 propagules mL^{-1} as determined by MPN analysis (Cochran, 1950). The PGPR inoculum contained 10^8 CFU mL^{-1} and was supplied as fine grade (0.5-1.0 mm) pumice containing four different rhizobacterial species (*Rhizobium* strain IRBG74, *Derxia lacustris* HL-12, *Bacillus megaterium* and *B. amyloliquefaciens*). In each experiment, the negative control (Cb$^-$) was not pre-inoculated or inoculated at planting. At the time of transplanting the granular AMF or PGPR inoculum was placed into each planting hole before transplantation of the strawberry plug. In Experiment 1 and 2, the volume of granular inoculum added per planting hole was ca. 7.6 mL for AMF and PGPR. In experiment 3, each pot received 25 mL of granular AMF inoculum. In Experiment 4, each planting hole received 20 mL of AMF inoculum and/or 2 mL of PGPR inoculum.

6.2.3 Pathogens inoculation

A mixture of three *P. fragariae* isolates (BC-1, BC-16 and Nov-9), from the pathogen collection of East Malling Research (Kent, UK) was used to inoculate plants in Experiment 1 and 2. The isolates were cultured separately in Petri dishes on sterile modified kidney bean agar (KBA) for at least 30 d in the dark at 18 ± 1 °C (Wynn 1968, Maas 1972). One piece (1-4 mm^2) of colonised KBA (from each stock culture) was then transferred to new Petri dishes containing fresh KBA and incubated as above. Mycelia were then harvested 30-60 d after plate inoculation. The excised colonies of the three isolates (including the agar beneath) were put into a blender with ice H_2O (1 g culture: 1 g ice H_2O) in equal ratio and blended twice for 2-5 s (19 × 103 rpm). The resulting inoculum slurry was transferred to a cooled beaker, which was kept on ice during the entire inoculation procedure. Before transplantation, roots were gently washed with tap water to remove substrates particles. Plants were then inoculated by dipping the roots into the inoculum slurry.

One *P. cactorum* isolate (P414; known to be pathogenic against 'Malling Centenary') from the pathogen collection of East Malling Research was used in Experiment 3. The stock culture was cultured in Petri dishes on a sterile V8 agar for 7 d in the dark at 18 ± 1 °C (Harris et al. 1997). Then a sterilised cork borer was used to cut 10 mm discs from the margins of actively growing cultures. Discs were immersed in a non-sterile compost extract (2 L distilled water drained through 50 g compost and diluted two fold before usage) and incubated for 2 d at 20 °C in an illuminated incubator. A suspension of 10^3 zoospores mL^{-1} was then produced following the method described by Harris *et al.* (1997). A vertical slit (ca. 10 mm long) was made using scalpel blade at the base of an internal leaf (close to the crown). The inoculum

was then directly sprayed onto the wounded area using a garden sprayer, 5 mL per plant. Inoculated plants were placed into a chilled glasshouse compartment (ca. 20 °C) and covered with a clear polythene sheet for 48 h to prevent the zoospores from drying out.

6.2.4 Transplantation

In Experiment 1, there were four treatments: plug inoculated at planting with (1) AMF [M], (2) PGPR [P], (3) both AMF and PGPR [M+P], and (4) a negative control with no microbes inoculated [Cb^-]. Each treatment was contained 20 replicates giving 80 plants in total and set-up in a randomised block design. About three weeks prior the start of the experiment, ca. 100 cold stored (-2 °C) plugs of cv. 'Vibrant' were transferred to a poly-tunnel with under natural temperature and light conditions for ca. two weeks to induce plant growth. Plants were watered once a day with tap water. No additional fertiliser was added.

All plants were inoculated with *P. fragariae* as described in Section 6.2.3. Immediately after pathogen inoculation, the plants were transplanted into 500 mL plastic pots (9 x 9 x 10 cm, Desch Plantpak Ltd, Essex, UK) filled up with ca. 400 mL of autoclaved (two cycles at 121 °C for 20 min with about 4 d between cycles) sandy compost (Table 6.2). The potting mix consisted of 1 part of sand (Sinclair horticulture Ltd, Lincoln, UK) and one part of sieved compost (reduced peat mix with added bark and grit reduced peat fertilised with Multi-Mix® [12N-14P_2O_5-24K added at 1 kg m^{-3}] from Sinclair Pro, Cheshire, UK). Pots were placed in a growth cabinet (Meridian Refrigeration Ltd, Croydon, UK; constant 15 °C, ca. 72 % relative humidity (RH), light: dark 16 h/8 h, photosynthetically active radiation (PAR) of 40 µmol m^{-2} s^{-1}). Plants stood in shallow layer of water (2-7 mm) during the entire experiment. The experiment was terminated five weeks after transplanting.

In Experiment 2, there were eight inoculation treatments: plant plugs inoculated at the transplanting time with (1) AMF [M], (2) PGPR [P], and (3) both AMF and PGPR [M+P]; (4) AMF pre-inoculated plugs [PreM], AMF pre-inoculated plugs inoculated at planting time with (5) AMF [PreM+M], (6) PGPR [PreM+P], and (7) AMF and PGPR [PreM+M+P]. Finally, there was a negative control without AMF pre-inoculation and no microbial inoculum added at planting time [Cb^-]. For each treatment, there were 16 plants, giving a total of 128 plants. A randomised block design with four blocks was used. About two weeks prior the start of the experiment, ca. 320 cold stored (2 °C) plugs of cv. 'Malling Centenary' were transferred to a growth cabinet (Meridian Refrigeration Ltd, Croydon, UK; day and night 15 °C, ca. 72 % RH, light: dark 16 h/8 h, PAR of 35 µmol m^{-2} s^{-1}) to induce plant growth. Plants were watered once a week with tap water and no additional fertiliser was added. Finally, plugs were inoculated with *P. fragariae* and treated as in experiment 1. The experiment was terminated after six weeks.

In Experiment 3, there were two treatments: (1) plug pre-inoculated with AMF [PreM] and (2) negative control without AMF pre-inoculation [Cb^-], each with 20 replicate plants, giving a total

of 40 plants. This experiment was conducted on two separate occasions (Table 6.1). Before pathogen inoculation, cold stored (-2 °C) plugs of cv. 'Malling Centenary' were transplanted in 500 mL plastic pots (9 × 9 × 10 cm, Desch Plantpak Ltd, Essex, UK) filled up with ca. 450 mL of standard compost mix (reduced peat mix with added bark and grit, fertilised with Multi-Mix® [12N-14P_2O_5-24K added at 1 kg m^{-3}] and Osmocote® [15N-9P_2O_5-11K added at 4.44 kg m^{-3}], from Sinclair Pro, Cheshire, UK; Table 6.2). Plants were then transferred to a poly-tunnel under natural temperature and light conditions for ca. five weeks to induce plant growth and AMF colonisation. All plants were then inoculated with *P. cactorum* as described in Section 6.2.3. Finally, each plant was randomised placed into a chilled glasshouse compartment (temperature set at 20 °C during the day and 15 °C during the night, with natural light cycle). Plants were manually watered once a day with tap water and no additional fertiliser was added. The experiment ran for seven weeks before destructive sampling.

In Experiment 4, plant plugs were inoculated at the planting time with (1) AMF [M], (2) PGPR [P] and (3) both AMF and PGPR [M+P]. There was also a negative control with no inoculum added [Cb^-]. This experiment was conducted on two separate occasions, with three replicates of each treatment each time (Table 6.1). A random design was used. Cold stored (-2 °C) plugs of cv. 'Malling Centenary' were planted in coir bags (BotaniCoir, London, UK), 10 plants per bag and inoculated with AMF and/or PGPR at planting as described in Section 6.2.2. Irrigation was delivered to plants via four irrigation lines using drippers (four per bag), controlled by Galcon irrigation timer (DC15, City Irrigation Ltd, Kent, UK). Each irrigation line alimented three coir bags. The duration of irrigation was adjusted over time based on plant growth, reached maximum at 6 weeks from plantation [peak of flowering] (1 L per day per bag). Concentred nutrient solution of Vitafeed 102 (100 g L^{-1}; Vitax Ltd, Leicester, UK) was injected in the irrigation lines by a dosatron injector (D3 Green line, City Irrigation Ltd, Kent, UK) set at a dose rate of 0.5 % for two weeks from plantation and then adjusted at a dose rate of 1 % and thereafter remained at this rate. After the onset of flowering a mini hive of bumblebees (*Bombus terrestris* audax; Agralan, Wiltshire, UK) was introduced to the compartment to pollinate flowers. Plants were grown in a glasshouse compartment set at 23 °C day/20 °C night with natural light cycle. Final destructive sampling was done after the final fruit harvest (ca. 13 weeks after planting).

Table 6.2. Background nutrient status analysisa (mg kg-1) of the substrate used in experiments.

Experiment	Substrate	pH	NO_3^-	NH_4^+	P	K	Mg
1	Sandy compost	5.8	75	27	16	200	45
2	Sandy compost	4.9	175	131	62	267	51
3	Compost	4.7	236	75	101	380	130
4	Coir	6.6	< 4	20	< 6	31	< 1

[a]Growth medium analysis was carried out by NRM Laboratories, Berkshire, UK. NO_3^- was determined by ion chromatography and NH_4^+ by colorimetric analysis. P, K and Mg was analysed by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy).

6.2.5 Disease assessment, plant productivity and root sample analysis

In Experiment 1, 2 and 3, plants were assessed once a week for aboveground disease symptoms on a rating scale: 1 – no symptoms, 2 – floppy foliage, and 3 – totally collapsed and dead.

In Experiment 3, after the final aboveground disease assessment, the crowns were cut longitudinally and the extent of internal necrosis was recorded: 1 – no necrosis, 2 – up to 25 % necrosis, 3 – 25 to 50 % necrosis, 4 – 50 to 75 % necrosis, and 5 – 75 to 100 % necrosis.

In Experiment 4, ripe fruits were picked twice weekly. Fruits were divided by size into Class 1 (> 18 mm diameter) and 2, weighed separately for individual coir bags and the number of fruit was recorded. After the final fruit harvest, total fresh shoot weight of the plants from individual bags was also determined. A few plants died during the experiment, thus for each bag average yield (total and class I), average number of fruit (total and class I), average fresh shoot weight per plant were calculated and used in subsequent statistical analysis.

At the end of each experiment, a composite sample of root was taken for each pot or coir bag (i.e. roots deriving from three plants out of 10 were pooled together) for assessment of AMF root colonisation preferentially sampling younger roots. The roots were then cleared with KOH before being stained with Trypan blue (Section 2.3.1) and microscopically assessed for root length colonisation (RLC; Section 2.3.2).

6.2.6 Data analysis

All data were analysed using GenStat 13th edition (VSN International Ltd, Hemel Hempstead, UK). Only significant differences are reported in the text. In Experiment 1-3, the disease data were all analysed using generalised linear models (GLM) with residual errors assumed to follow a binomial distribution; the log link function was used. There were two treatments factors (M and P) in Experiment 1, three factors in Experiment 2 (PreM, M and P) and one factor in Experiment 3 (M). The interactions among factors in Experiment 1 and 2 were included. In

Experiment 3, individual experiments conducted at different times were treated as a blocking factor. In Experiment 4, for each bag average fruit yields (total and Class I fruit), average number of fruits (total and Class I fruit) and average plant fresh shoot weight per plant were analysed by two-way ANOVA. Individual replicate experiments were treated as a blocking factor. Common diagnostic plots (e.g. q-q plots, residual-fitted value plot) did reveal apparent violation of the normality and homoscedasticity assumption. Hence, average number of fruit data were square root transformed, while average yield and fresh shoot weight were both log transformed to satisfy normality. If ANOVA indicated significant effects of a specific treatment factor or interaction, pairwise comparison was then performed based on the LSD test. In all experiments, the AMF root colonisation data were not statistically tested due to the absence or low level of colonisation observed within roots.

6.3 Results

6.3.1 Establishment of AMF in the substrates

The level of AMF colonisation of plant roots is generally very low. In Experiments 1-3 there was no AMF colonisation observed in the roots of AMF treated plants. In Experiment 2 AMF pre-colonised plugs that showed an average of 3 % RLC before transplantation were not colonised by the end of the experimental period. However, AMF colonisation in experiment 4 was observed albeit at a low level (average < 15 % RLC) and varied greatly among samples; there was no AMF colonisation in many samples. There were also no obvious differences in RLC between M and M+P treatments.

6.3.2 Effect of beneficial microbes against red core and crown rot

The effects of AMF and PGPR inoculations against *P. fragariae* (red core) were studied in Experiments 1 and 2. In Experiment 1, the first wilting symptoms appeared eight days after pathogen inoculation. In general, the number of plants with visible symptoms remained stable after 20 d from inoculation (Figure 6.2A, B); 60 out 80 plants showed disease symptoms at the end of the experiment. The number of diseased plants was decreased by AMF inoculation at planting (GLM, P = 0.020; Figure 6.3), while PGPR treatment did not affect the number of diseased plants. There was no significant interaction between AMF and PGPR.

Figure 6.2. Plants inoculated with *Phytophthora fragariae* in Experiment 1 (view of block 4 only) at two different time points: (A) at planting and (B) three weeks after planting. Diseased plants were wilted and showed brown leaves (red arrows).

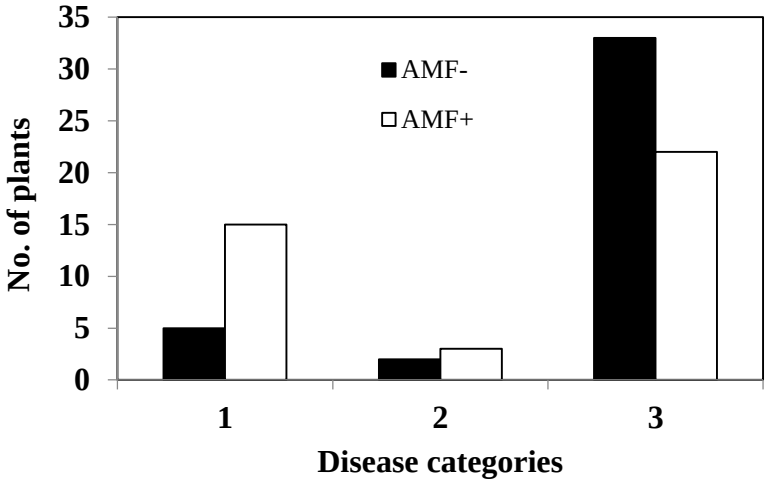

Figure 6.3. Influence of arbuscular mycorrhiza fungi (AMF) addition in experiment 1 on disease score 35 d after inoculation of *Phytophthora fragariae*. Data are the total number (*n* = 40) of plants in each of the following disease category: 1 – no symptoms, 2 – floppy foliage, 3 – totally collapsed and dead. AMF+ were plants inoculated with mycorrhiza at planting (i.e. M and M+P) and AMF- were plants without mycorrhiza (i.e. P and Cb^{-}).

In Experiment 2, the first wilting symptoms appeared in less than a week after inoculation. No more symptoms appeared after 20 d from red core inoculation and the overall number of wilted plants remained lower than in experiment 1; 40 out 128 plants showed disease symptoms. Diseased plants were evenly distributed in the four blocks of the experiment. However, there were no significant treatment effects (PreM, M, P) on the number of diseased plants. In no case was the interaction term among PreM × M × P statistically significant.

The effect of mycorrhizal inoculation against *P. cactorum* (crown rot) was studied in Experiment 3. In both replicate experiments, the first wilting symptoms appeared about two weeks after inoculation (Figure 6.4A) and the number of plants with visible symptoms remained stable after five weeks from inoculation. In total, 31 and 33 out of 40 plants showed crown necrosis for the 1st and 2nd replicate experiment, respectively (Figure 6.4B). However, there were no significant effects of AMF pre-inoculation on the number of diseased plants and crown necrosis level.

Figure 6.4. Plant inoculated with *Phytophthora cactorum* in Experiment 3 (the first repeat experiment) seven weeks after pathogen inoculation: (A) severely wilted plant and (B) observation of crown necrosis (level 4, see section 6.2.5).

6.3.3 AMF and PGPR on strawberry production in coir

Strawberry plants in experiment 4 grew normally and there were no visual differences in plant growth among treatments. A single plant in each coir bag produced on average 9 and 10 fruits for the 1st and 2nd repeat experiment, respectively; the corresponding average fruit weight was 60 and 67 g. There were no significant differences in the average plant yields (i.e. for total and class I fruit) and average plant number of fruits (i.e. for total and class I fruit) among treatments. The interaction involving the factors M and P was not statistically significant. For the average plant fresh weight, none of the treatments resulted in significant differences and there was no significant interaction. Despite the absence of significant effect, the average plant yields and number of fruits (total and class I) followed the same treatment order: P > M+P > M > Cb^-

(Figure 6.5A), whereas the treatment order for average plant shoot fresh weight was: M > M+P > P > Cb$^-$ (Figure 6.5B).

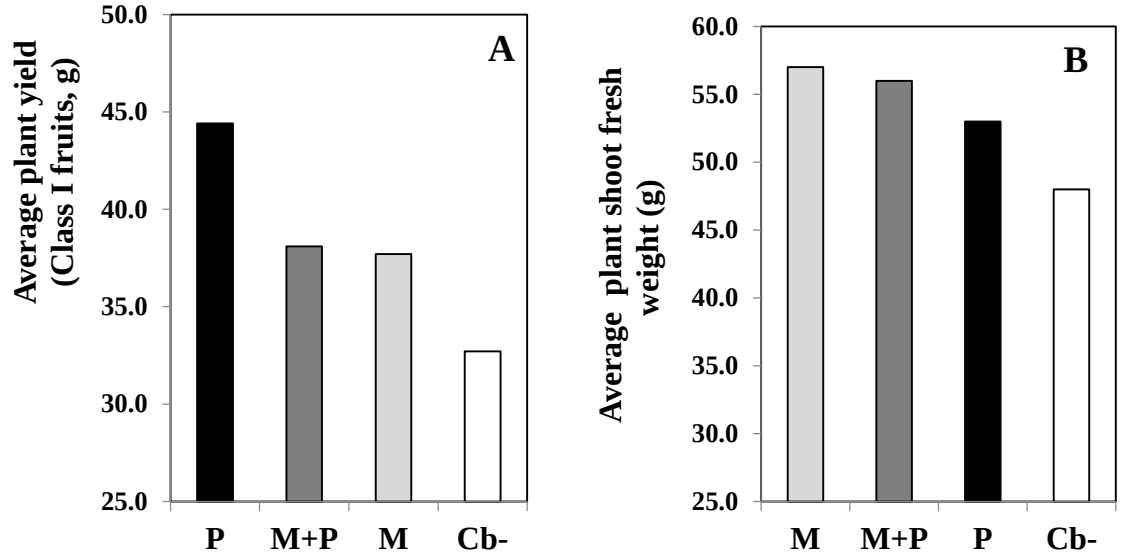

Figure 6.6. Average plant class I fruit yield (A) and average plant shoot fresh weight (B). The treatments were plugs inoculated at the planting time with AMF (M), PGPR (P), both AMF and PGPG (M+P) and a negative control with neither AMF nor PGPR added (Cb$^-$). [note – further more sophisticated analysis will be carried out trying to take into account the effect of irrigation lines]

6.4 Discussion

The soil-less substrates used in horticultural production usually lack beneficial microbes (Postma et al. 2008). By inoculating commercial AMF and PGPR alone or together to the rhizosphere, it might be possible to reduce chemical inputs and grow strawberry in more sustainable way (Boyer et al. 2016). Bacteria and mycorrhizal fungi occurring naturally in plant rhizosphere could be excellent candidates for the development of a biocontrol agent or a biofertiliser because they are already part of the balance between plants, pathogens and soil (Whipps 2004). Several issues related to the plant-microbe-substrate interactions need to be studied and understood before AMF and PGPR could be successfully used for commercial agriculture. The main objective of this study was to investigate whether commercially available AMF and/or PGPR inoculants could reduce development of *P. fragariae* (red core) and *P. cactorum* (crown rot) in peat-based composts and improve strawberry productivity in coir under controlled conditions.

In Experiment 1-3, AMF inoculation in composts mixes containing peats and fertilisers did not result in strawberry root colonisation. In contrast, low level of root colonisation (< 15 %) was detected in coir bags in Experiment 4. Therefore, soil-less substrates used in this study

appeared not to be conducive for AM root colonisation. The reasons for this are unknown. Strawberries have been described as a mycotrophic plant species, which are usually well colonised in soil (Vestberg 1992b, Vestberg et al. 2000). Nevertheless, previous studies reported negative effects of certain peat types and coir on strawberry RLC (Vestberg et al. 2004b, Boyer et al. 2016). In addition, high inputs of fertilisers under commercial practices have been shown to reduce strawberry root colonisation (Niemi and Vestberg 1992). Consequently, it is unclear whether the absence of root colonisation in the compost mixes and very low RLC observed in coir were the results of high concentration of available P or the combination of chemicals, physical and biological properties of the substrates themselves. However, there were rather high levels of P in the compost mixes used in Experiment 1-3 compare to the coir used in Experiment 4 (Table 6.2). Fertilised compost mixes were previously used to test the effect of *Phytophthora* on strawberry plants (Murphy et al. 2000b, Vestberg et al. 2004b). Therefore, the absence of AMF colonisation in the compost mixes may have been partly due to the high level of available P. Further investigations on the role of commercial fertilisers and substrates properties on AMF colonisation are needed.

In Experiment 1, the number of diseased plants was reduced by AMF inoculation at planting time. However, PGPR alone and co-inoculation with AMF did not result in reduced disease development. This agrees with several studies highlighting the positive effect of AMF inoculation against red core (Mark and Cassells 1996, Norman et al. 1996b, Norman and Hooker 2000). In contrast, Vestberg *et al.* (2004) reported an increase in disease development of *P. fragariae* after AMF inoculation (alone or in mixture with PGPR). However, it should be noted that although the AMF inoculation showed a positive effect against red core in Experiment 1, AMF colonisation was not detected in the roots. Either the sampling method failed to detect a low level of AMF colonisation or the substrate containing the AMF inoculum (i.e. attapulgite clay/pumice/zeolite mix) could have achieved this as attapulgite clay has previously been found to limit the development of *Phytophthora* symptoms in strawberry plants (Hautsalo et al. 2016). In Experiment 2, neither AMF pre-inoculation, nor microbial inoculations at planting, nor the combination of both inoculation methods reduced disease development. Fewer plants in Experiment 2 suffered from red core (31 %) than in Experiment 1 (75 %). It is, however, unclear whether the lower level of diseased plants in Experiment 2 was due to the differences in susceptibility between the two cultivars and/or an effect of the growing season. Previous studies have highlighted the difference of red core susceptibility between strawberry cultivars (Van de Weg 1997) and the growing season has also been shown to modify the effect of the beneficial microbes against *Phytophthora* (Vestberg et al. 2004b). The reasons for the difference in disease development may be the physiological status of the strawberry hosts that may modify the root exudation and the microbial community composition in the rhizosphere, either introduced or natural. In Experiment 3, disease reduction was also not achieved by AMF

pre-inoculation during the season favourable for strawberry growth. The reason for this is not known. Nevertheless, in Experiment 2 and 3, the absence of AMF colonisation in the strawberry roots might explain the absence of biological control effect.

Inoculation of plants with AMF and/or PGPR at planting time in coir had a consistent positive effect on shoot fresh weight, fruit yield and number of fruits produced compare to the control without inoculation of beneficial microbes. These observations agree with previous reports of AMF inoculation of strawberry in coir (Boyer et al. 2016). Co-inoculation of AMF and PGPR did not give better results than the inoculation of either AMF or PGPR alone for strawberry growth and yield, agreeing with a previous study (Vestberg et al. 2004b). However, the positive effects of AMF and PGPR observed in the current study were not statistically significant. The nature of the experimental design (i.e. complete randomisation design) could have been responsible for large experimental residual errors since it did not explicitly consider the potential large differences between irrigation lines. Four irrigation lines were used for this study to deliver water into coir substrate. Further sophisticated analysis will be tried to see whether the irrigation line effect could be taken into consideration.

Conclusions

1. AMF propagules can colonise strawberry roots when incorporated as a powder layer in an Irish peat/base mix, vermiculite and coir under misting conditions or plant propagators.
2. There is no significant association between strawberry cultivars and AMF strains/species in terms of the extent of root colonisation by AMF
3. AMF in the form of colonised roots and/spores can survive several months of cold storage at -2 °C in tray plants.
4. Pre-inoculation of strawberry tipping plants at the weaning stage with AMF does not necessarily improved plant growth during weaning and subsequent early growth before cold storage.
5. The present study highlighted the difficulty to induce wilt symptoms in pot under glasshouse conditions when using field soil with a high level of wilt inoculum. When planted in field, AMF pre-inoculation failed to reduce the incidence wilt, compared to the control plants. Thus, for field production pre-colonisation plants with AMF may not provide additional advantages since there may be sufficient amount of resident AMF in soils.
6. AMF and PGPR could reduce development of Phytophthora diseases in coir. However, this effect is not consistently observed over replicate experiments. Furthermore, although the increased tolerance is statistically significant in one study, such an increase may not offer any commercial value. Thus, we may conclude that using AMF and PGPR alone will not be able to reduce disease development particularly when the plants were either already infected or inoculated with a high dose of pathogen inoculum.
7. AMF and PGPR used alone appear to increase strawberry yield by 5-10%. However, inoculation at the transplanting stages (whether bare-runners or tray plants) did not always result in **consistently** high level of colonisation, which may explain the lack of observed effects.
8. Random mix of AMF inocula with compost in the commercial production of tipping plants resulted in a very low level of AMF colonisation. Thus, if AMF is to be incorporated at this stage, some modification may need to be applied to the commercial tipping process to ensure that AMF is present as a layer just below the surface of compost in each cell. It would increase the likelihood of AMF colonisation of new roots during misting/weaning.

Knowledge and Technology Transfer

1. Sept 2014. Can arbuscular mycorrhizal fungi control strawberry wilt? Poster presentation at the Postgraduate Fair, University of York
2. Sept 2014. Pre-colonisation of strawberry runners and tray plants with arbuscular mycorrhizal fungi to manage *Verticillium* wilt. Poster presentation at the HDC PhD Student conference
3. March 2015. Arbuscular mycorrhizal fungi pre-inoculation for improving the growth and health of strawberry planting materials. Oral presentation to the PhD student journal club at NIAB EMR
4. August 2015. Arbuscular mycorrhizal fungi pre-inoculation for improving the growth and health of strawberry (*Fragaria* x *ananassa*) planting materials. Poster presented at The Eighth International Conference on Mycorrhiza (ICOM8): Mycorrhizal integration across continents & scales, August 3–7, 2015, Northern Arizona University, Flagstaff, Arizona, USA
5. Sept 2015. Arbuscular mycorrhizal fungi pre-inoculation for improving the growth and health of strawberry planting materials. Oral presentation at the SCI Horticulture Group AGM
6. Sept 2015. Pre-colonisation of strawberry runners and tray plants with arbuscular mycorrhizal fungi to manage Verticillium wilt. Poster presentation at the HDC PhD Student conference
7. May & July 2015. Discuss with a UK commercial nursery about producing pre-colonisation of tipping plants using the commercial production process.
8. Sept 2015. Establishment of arbuscular-mycorrhizal fungi on tissue culture derived strawberry to improve plant development and tolerance to *Verticillium* wilt. Poster presentation at the Postgraduate Fair, University of York
9. July 2014. Arbuscular mycorrhizal fungi pre-colonisation for improving the growth and health of strawberry (*Fragaria* x *ananassa*). Poster presented at the SCI Young Researchers in Crop Sciences 2016, Syngenta, Bracknell
10. Aug 2016. Arbuscular mycorrhizal fungi pre-colonisation for improving the growth and health of strawberry (*Fragaria* x *ananassa*). Poster presentation at the 16th International Society of Microbial Ecology (ISME), Montreal, Canada

Glossary

- AMF: arbuscular mycorrhizal fungi
- PGPR: Plant growth-promoting rhizobacteria

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